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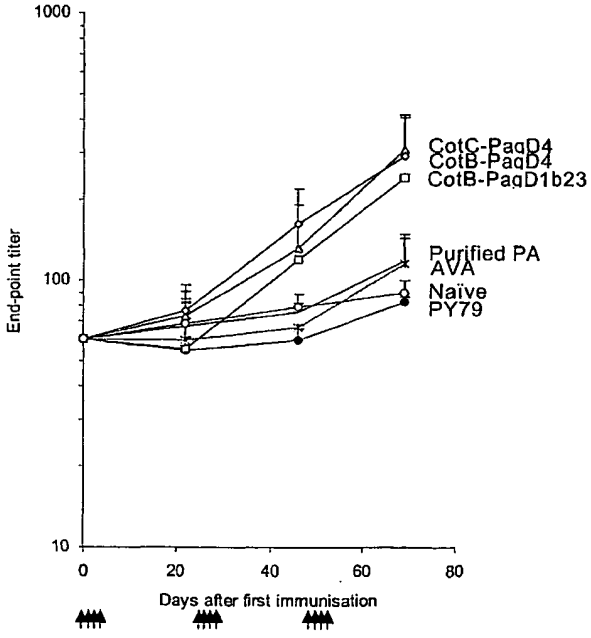
(54) Title: ANTHRAX VACCINE IN THE FORM OF A SPORE

(57) Abstract: The invention provides a non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen for use as an anthrax vaccine particularly by nasal and/or oral administration.



ELISA titers *vs* time for Nasal Immunisation with spore coat expression constructs

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ANTHRAX VACCINE IN THE FORM OF A SPORE

The present invention relates to a new vaccine for anthrax, a method of inducing an immune response against anthrax and a method of preparing an anthrax vaccine.

Anthrax is an acute infectious disease caused by the spore forming bacterium *Bacillus anthracis*. The disease takes three forms in humans; cutaneous, gastrointestinal and inhalation (pulmonary anthrax) with the gastrointestinal and inhalation forms being the most deadly with mortality rates of up to 90%. The anthrax bacterium is found globally, present in the soil as extremely resistant spores and most commonly causes disease in hoofed animals. There are an estimated 20,000 to 100,000 human cases of anthrax per year, most of which occur in the developing world. However, in the US, the annual incidence had declined to less than 1 case per year. The recent use of anthrax as a bioterrorism agent in the US in October to December 2001 clearly demonstrated the potential of this bacterium as a biowarfare agent with 12 cases of clinically confirmed anthrax documented. These included 6 cases of inhalation anthrax (3 fatal) and 6 cases of cutaneous anthrax. Anthrax is currently recognised alongside smallpox as the most likely biological warfare agent by Western governments.

The current veterinary vaccine is a spore preparation of a *B. anthracis* strain (the Sterne strain) lacking the pXO2 plasmid. This strain produces anthrax toxin but is unable to synthesise the capsule required for survival in host cells. While effective this veterinary vaccine has a low level of virulence (since it still produces the anthrax toxin) and can produce necrosis at the site of injection (Hambleton & Turnbull, 1990). In contrast, the human vaccine is a non-cellular vaccine (AVA) produced in the UK and USA from a toxigenic, non-capsulated, Sterne strain by

adsorbing culture supernatant to aluminium hydroxide. The key component of this vaccine is the 82.7 kDa protective antigen (PA). While efficacy has been established in various animal models, vaccination requires multiple doses and boosters (six injections over 18 months). Adverse side effects have been reported (erythema, induration) and in some animals the vaccine does not provide protection against all virulent *B. anthracis* strains (Hambleton & Turnbull, 1990). Finally the AVA vaccine is expensive to produce. Recent work has shown that anthrax vaccination is greatly enhanced if *B. anthracis* spores of the Sterne strain are included in the vaccine together with PA and that the spores themselves play a critical role in stimulating protective immunity (Brossier et al., 2002).

Protective antigen (PA; mwt. 82.7 kDa) is the binding/translocating component of both toxins (LeTx and EdTx) produced by *B. anthracis* and encoded by the *pag* gene. Administered alone, PA is not toxic but must associate with lethal factor (LF) to produce LeTx and edema factor (EF) to produce EdTx. PA is secreted from the *B. anthracis* cell and is involved in receptor binding to host cells and then for translocation of PA-EF and PA-LF to the cell cytoplasm. PA is protective, that is, immunisation of an animal model with this protein can alone provide protection to challenge with *B. anthracis*.

Current vaccine strategies require provision of protective antigen to the vaccinee using either purified PA or purified PA plus attenuated/inactive spores of *B. anthracis*.

Support for new vaccine development is provided primarily by defence and antiterrorism measures within national programmes in the UK e.g. the Ministry of Defence and the Department of Defence, often supported

by national health research agencies such as the Medical Research Council and the National Institute of Health.

Many pathogens are able to initiate disease through infection of the mucosal surfaces. The infectious agent first makes contact with, and then colonises (or transverses), the mucosal surface to infect the host (e.g., HIV, TB). Traditional vaccination strategies relying on parenteral immunisation are unable to prevent the initial interaction of pathogen and host at the mucosal surface but rather resolve the resulting infection (Walker, 1994). Oral (or intranasal) immunisation should induce secretory IgA antibodies directed against the specific pathogen as well as CTL responses from CD8+ MHC restricted cytotoxic T-lymphocytes located in the mucosal epithelium and draining lymph nodes (LN) providing an effective means for preventing infection. One problem with oral immunisation, though, is that orally administered antigens frequently lose their immunogenicity. These limitations can be countered using attenuated and live bacteria (e.g. *Salmonella* spp.) that act as carriers of heterologous antigens. One of the most pervasive limitations to the use of bacteria as vaccine vehicles though is their lack of heat stability. A vaccine carrier that can induce mucosal immunity, that can be used orally or nasally and is heat-stable is obviously attractive for development of the next phase of improved vaccines. Further improvements in vaccines against anthrax have been sought.

According to the invention there is provided a non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen.

According to the invention there is also provided a pharmaceutical composition comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in association with a pharmaceutically acceptable carrier and/or excipient.

According to the invention there is further provided a spore according to the invention for use as an anthrax vaccine.

According to the invention there is also provided a pharmaceutical composition according to the invention for use as an anthrax vaccine.

According to the invention there is further provided use of a spore according to the invention or of a pharmaceutical composition according to the invention in the manufacture of a medicament for use as an anthrax vaccine.

According to the invention there is further provided use of a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in the manufacture of a vaccine for anthrax for non-parenteral administration, preferably nasal and/or oral administration.

According to the invention there is further provided a method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises non-parenteral administration, preferably nasal and/or oral administration of an effective amount of a vaccine comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen

According to the invention there is further provided a method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises administration of an effective amount of a vaccine comprising a spore according to the invention or a composition according to the invention to the mammal.

Advantages of the invention include that it is not necessary to use a live attenuated *B. anthracis* strain. This is advantageous for ethical reasons where it is not acceptable, long term, to be using an attenuated pathogen and enables the vaccine to be used with vaccines having a compromised immune response. A further advantage is that *B. subtilis* which is a preferred source of a spore according to the invention is relatively easy and cheap to produce.

Another advantage is that the invention provides a spore-specific immune response to anthrax which is important because this is one way in which anthrax is transmitted. Even though the spores used in the invention are non-pathogenic and therefore not of *B. anthracis*, they will still generate a spore-specific response, enhancing the efficacy of protection. Anti-PA antibodies have been shown to stimulate phagocytosis of *B. anthracis* spores while inhibiting their germination within the phagolysosome. This appears to be an efficient method for dealing with pathogenic spores. How spore germination is inhibited is unclear but presumably binding of anti-PA antibodies to the spore prevents entry of germinants. It has been proposed that the phagosome/phagolysosome somehow provides a germination signal (Guidi-Rontani *et al*, 1999).

A further advantage to the invention is that it has been found that the spore according to the invention germinates within a phagocyte. It is known that *B. anthracis* spores germinate in macrophages which is critical for pathogenesis and contributes to cellular responses (Th1 dependant) as well as enhancing humoral immune responses (probably TH2 dependant). Thus it is believed that this ability of spores to persist in macrophages is a common phenomenon to *Bacillus* spores and serves to elicit cellular responses. Accordingly the use of a non-pathogenic spore for antigen delivery mimics the fate of *B. anthracis* spores and enhances

vaccine potency. Furthermore it has surprisingly been found that a spore according to the invention can be used to generate mucosal immunity.

The non-pathogenic spore is preferably from a *Bacillus* species spore. More preferably the spore is from one or more of the following organisms: *Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis; Bacillus mycoides; Bacillus pasteurii; Bacillus sphaericus; Bacillus aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus freudenreichi; Bacillus macroides; Bacillus similibadius; Bacillus thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis; Bacillus bengalicus; Bacillus flexus;* and/or *Bacillus licheniformis*. Most preferably, the spore is from *Bacillus subtilis*.

The spore is non-pathogenic. This generally means that neither the spore nor a bacterium into which the spore may germinate is harmful to the host to which the spore is to be administered.

B. subtilis is a ubiquitous, Gram positive, non-pathogenic organism, normally found in the soil. The spore is a dormant life form that can resist extreme environmental conditions (Nicholson et al., 2000) and has a number of attributes making them particularly suitable for the development of a generic vaccine system, these are:-

- Dormant with suitable storage and desiccation properties,
- Suitable for non-parenteral delivery, particularly by the oral and nasal route,
- No evidence for compromised immune status in man,
- Can be deactivated, e.g. with gamma radiation, if necessary,
- Easily modified genetically,
- Can be produced in large quantities; safely, efficiently and cost effectively,

- Robust, can survive indefinitely at temperatures up to 90°C,
- Suitable for field use, particularly in developing countries,
- Resistant to UV irradiation and desiccation,
- Currently used in Europe as a probiotic for human use.

The spore is optionally either a germinating spore or a non-germinating spore. Where the spore is non-germinating, it has preferably been treated to prevent germination. Germination can be prevented by using gamma irradiation or by using a germination-deficient mutant spore (such as that disclosed in Duc *et al.*, 2003a).

The spore is preferably a germinating spore. This is because it has been found that an improved immunogenic response can be obtained with a vaccine using such a spore.

Where the non-pathogenic spore used in the invention comprises at least an antigenic fragment of anthrax protective antigen, it is to be understood that it comprises either anthrax protective antigen or a fragment thereof. The antigenic fragment of anthrax protective antigen used in the invention is generally a fragment of anthrax protective antigen which is sufficient to stimulate a suitable immunogenic response.

The spore may optionally comprise at least an antigenic fragment of anthrax protective antigen in the form of a protein attached to the spore (preferably the at least an antigenic fragment is attached to the proteinaceous coat of the spore) and/or in the form of DNA which encodes at least an antigenic fragment of anthrax protective antigen which DNA is adapted to be expressed when the spore germinates.

Where the at least an antigenic fragment of anthrax protective antigen is provided in the form of DNA, the DNA is preferably under the control of

a vegetative cell promoter so that the DNA is only expressed when the spore germinates and/or begins to outgrow. A suitable promoter is for example rrnO.

In the present invention, the at least an antigenic fragment of anthrax protective antigen is preferably either PA83 (full length) (SEQ ID No. 16) or a fragment thereof. A fragment of PA83 (full length) is preferably one or more of PA83 (SEQ ID No. 6), PA63 (SEQ ID No. 7), Domain 1 of PA (SEQ ID No. 1), Domain 2 of PA (SEQ ID No. 2), Domain 3 of PA (SEQ ID No. 3), Domain 4 of PA (SEQ ID No. 4) and Domain D1b23 of PA (SEQ ID No. 10).

There are two optimal routes for expression on a spore which can be used which are fusion of the DNA encoding the at least an antigenic fragment of anthrax protective protein and/or a truncated form to CotB or CotC with a suitable promoter for CotB or CotC. In each case genetic engineering is used to splice the *cotB* or *cotC* genes to the *B. anthracis* sequences encoding PA83 (full length) or a fragment thereof.

To express the antigenic fragment of anthrax protective antigen in a germinating spore, the same sequences are fused to an expression cassette.

The sequence of a promoter used in the invention is SEQ ID No. 5. It allows convenient insertion of any ORF (open reading frame) downstream of strong transcriptional and translational signals. The preferred transcriptional signals are comprised of the -35 and -10 promoter sequences of the *rrnO* gene of *B. subtilis*.

The translational signals are provided by placing the start codon and ribosome binding site of the *sspA* gene of *B. subtilis*. *sspA* encodes a

small acid soluble protein that is expressed during sporulation. Expression using this RBS is high.

The sequence of the chimeric promoter Prrn0-RBS(sspA) is shown in Figure 15 and arrangements shown schematically in Figs 2 and 3. Immediately downstream of the ATG start codon is a multiple cloning site (MCS) carrying numerous restriction endonuclease sites. The MCS was derived from the pET vector pET28b (Novagen).

Any ORF sequence generated by PCR or other means can be cloned into the MCS in such a way as to allow in frame fusion of the inserted ORF with the start codon.

In the invention, three cloning vectors were used which are pDL242 (6.3 kbp) (Figure 2), pDL243 (6.3 kbp) (Figure 3) and pDG364 that carry the Prrn0-RBS(sspA)-MCS cassette.

pDL242 (Figure 2) is derived from the plasmid pDG1663 (Guerout-Fleury et al., 1996). pDG1663 allows insertion of foreign DNA into the *B. subtilis* chromosome by what is referred to as a double crossover recombination or marker replacement as shown in Figure 4 and described in (Guerout-Fleury et al., 1996). Integration occurs only at the *thrC* locus. pDL242 carries the *erm* gene that renders transformed cells resistant to erythromycin.

To use this plasmid, a DNA is inserted at the MCS site of pDL242. Ligated molecules are transformed into *E. coli* with selection for Amp^R (ampicillin resistance) and recombinants are screened using PCR analysis of plasmids. Plasmid clones are then prepared in *Escherichia coli* and plasmid DNA clone verified by sequencing across the site of the fusion junctions. Next, the plasmid is linearised by restriction digest using sites

cutting in the backbone of pDL242 (see (Guerout-Fleury et al., 1996)) and DNA transformed into competent *B. subtilis* with selection for Erm^R. PvuI is the preferred enzyme for linearisation of pDL242. Transformants can only arise if a double crossover recombination has occurred between homologous segments of the *thrC* gene carried on the host chromosome and the pDL242 clone.

Transformants are checked to ensure they are Erm^R and $ThrC^-$ (since integration at the *thrC* locus will destroy the gene destroying the ability of cells to grow without added threonine).

Cells are then cultured and expression of the gene product cloned into the cassette verified by Western blotting, dot-blotting quantification and immunofluorescence microscopy.

pDL243 is shown in Figure 3 and is similar to pDL242. The vector is derived from the plasmid pDG364 (Cutting and Vander-Horn, 1990; Karmazyn-Campelli et al., 1992). pDG364 allows insertion of foreign DNA into the *B. subtilis* chromosome by what is referred to as a double crossover recombination or marker replacement as shown in Figure 5 and described in (Cutting and Vander-Horn, 1990; Guerout-Fleury et al., 1996). Integration occurs only at the *amyE* locus. pDL243 carries the *cat* gene that renders transformed cells resistant to chloramphenicol.

To use this plasmid, a DNA is inserted at the MCS site of pDL243. Ligated molecules are transformed into *E. coli* with selection for Amp^R (ampicillin resistance) and recombinants are screened using PCR analysis of plasmids. Plasmid clones are then prepared in *Escherichia coli* and plasmid DNA clone verified by sequencing across the site of the fusion junctions. Next, the plasmid is linearised by restriction digest using sites cutting in the backbone of pDL243 (see (Cutting and Vander-Horn,

1990)) and DNA transformed into competent *B. subtilis* with selection for Cm^R. PvuII is the preferred enzyme for linearisation of pDL243. Transformants can only arise if a double crossover recombination has occurred between homologous segments of the *amyE* gene carried on the host chromosome and the pDL243 clone as shown in Figure 5.

Transformants are checked to ensure they are Cm^R and $AmyE^-$ (since integration at the *amyE* locus will destroy the gene destroying the ability of cells to grow without added threonine).

Cells are then cultured and expression of the gene product cloned into the cassette verified by Western blotting, dot-blotting quantification and immunofluorescence microscopy.

Regarding the translational start signals (RBS = ribsome binding site or Shine Dalgarno (SD) sequence, the optimum rbs is AAAGGAGGTGA and the *sspA* RBS has AAGGAGGTGA. In principle the rbs could be taken from any gene or made synthetically.

The pharmaceutical composition according to the invention comprises a spore according to the invention and a pharmaceutically acceptable carrier and/or excipient. Processes for manufacturing a pharmaceutical composition are well known. The components of the composition may be combined with any combination of optional additives (e. g., at least one diluent, binder, excipient, stabilizer, dessicant, preservative, coloring, or combinations thereof). See, generally, Ullmann's Encyclopedia of Industrial Chemistry, 6th Ed (electronic edition, 1998); Remington's Pharmaceutical Sciences, 22nd (Gennaro, 1990, Mack Publishing); Pharmaceutical Dosage Forms, 2nd Ed. (various editors, 1989-1998, Marcel Dekker); and Pharmaceutical Dosage Forms and Drug Delivery Systems (Ansel et al., 1994, Williams & Wilkins).

A pharmaceutical composition according to the invention may be in the form of an cream, emulsion, gel, lotion, ointment, paste, solution, suspension, or other liquid forms known in the art. A pharmaceutical composition according to the invention may optionally also comprise an adjuvant which potentiates an antigen-specific immune response.

A sterile liquid composition suitable for use as the pharmaceutical composition according to the invention may be prepared by suspending an intended component of the formulation in a sufficient amount of an appropriate sterile solvent. Generally, dispersions are prepared by incorporating the various sterilized components of the formulation into a sterile vehicle which contains the basic dispersion medium. For production of solid forms that are required to be sterile, vacuum drying or freeze drying can be used. Solid dosage forms (e. g., powders, granules, pellets, tablets) or liquid dosage forms (e. g., liquid in ampules, capsules, vials) can be made from at least one active ingredient or component of the formulation.

The relative amounts of active ingredients within a dose and the dosing schedule may be adjusted appropriately for efficacious administration to a subject (e.g., animal or human). This adjustment may depend on the subject's particular disease or condition, and whether therapy or prophylaxis is intended. To simplify administration of the formulation to the subject, each unit dose would contain the active ingredients in predetermined amounts for a single round of immunization.

There are numerous causes of protein instability or degradation, including hydrolysis and denaturation. In the case of denaturation, a protein's conformation is disturbed and the protein may unfold from its usual globular structure. Rather than refolding to its natural conformation,

hydrophobic interaction may cause clumping of molecules together(i.e., aggregation) or refolding to an unnatural conformation. Either of these results may entail diminution or loss of antigenic activity. A stabilizer may be added to lessen or prevent such problems.

The pharmaceutical composition according to the invention, or any intermediate in its production, may be pretreated with protective agents (i.e., cryoprotectants and dry stabilizers) and then subjected to cooling rates and final temperatures that minimize ice crystal formation. By proper selection of cryoprotective agents and the use of preselected drying parameters, almost any formulation might be cryoprepared for a suitable desired end use.

It should be understood in the following discussion of optional additives like excipients, stabilizers, dessicants, and preservatives are described by their function. Thus, a particular chemical may act as some combination of excipient, stabilizer, dessicant, and/or preservative. Such chemicals would be considered immunologically inactive because it does not directly induce an immune response, but it increases the response by enhancing immunological activity of the antigen or adjuvant: for example, by reducing modification of the antigen or adjuvant, or denaturation during drying and dissolving cycles.

Stabilizers include cyclodextrin and derivatives thereof (see U. S. Patent 5,730,969). Suitable preservatives such as sucrose, mannitol, sorbitol, trehalose, dextran, and glycerin can also be added to stabilize the final formulation. A stabilizer selected from nonionic surfactants, D-glucose, D-galactose, D-xylose, D-glucuronic acid, salts of D-glucuronic acid, trehalose, dextrans, hydroxyethyl starches, and mixtures thereof may be added to the formulation. Addition of an alkali metal salt or magnesium chloride may stabilize a polypeptide, optionally including serum albumin

and freeze-drying to further enhance stability. A polypeptide may also be stabilized by contacting it with a saccharide selected from the group consisting of dextran, chondroitin sulfuric acid, starch, glycogen, insulin, dextrin, and alginic acid salt. Other sugars that can be added include monosaccharides, disaccharides, sugar alcohols, and mixtures thereof (e. g., glucose, mannose, galactose, fructose, sucrose, maltose, lactose, mannitol, xylitol). Polyols may stabilize a polypeptide, and are water-miscible or water-soluble. Suitable polyols may be polyhydroxy alcohols, monosaccharides and disaccharides including mannitol, glycerol, ethylene glycol, propylene glycol, trimethyl glycol, vinyl pyrrolidone, glucose, fructose, arabinose, mannose, maltose, sucrose, and polymers thereof. Various excipients may also stabilize polypeptides, including serum albumin, amino acids, heparin, fatty acids and phospholipids, surfactants, metals, polyols, reducing agents, metal cheating agents, polyvinyl pyrrolidone, hydrolyzed gelatin, and ammonium sulfate.

Single-dose formulations can be stabilized in poly (lactic acid) (PLA) and poly (lactide-co-glycolide) (PLGA) microspheres by suitable choice of excipient or stabilizer. Trehalose may be advantageously used as an additive because it is a nonreducing saccharide, and therefore does not cause aminocarbonyl reactions with substances bearing amino groups such as proteins.

The invention is illustrated with reference to the following Figures of the accompanying drawings:

Figure 1 shows schematically the role of the *Bacillus anthracis* protein Protective Antigen;

Figure 2 shows the construction of vector DL242;

Figure 3 shows the construction of vector DL243;

Figure 4 shows the integration of vector DL242 into *B. subtilis* chromosome;

Figure 5 shows the integration of vector DL243 into *B. subtilis* chromosome;

Figure 6 shows single constructs of PA63 and Domain 4 of PA with CotB and a CotB promoter;

Figure 7 shows single constructs of PA63 and Domain 4 of PA with CotC and a CotC promoter;

Figure 8 shows single constructs of PA83, PA63 and Domain 4 of PA with *rrnO* promoter;

Figure 9 shows Western blots specific for PA wherein PY79, non-recombinant *B. subtilis*. Spore coat extracts are fractionated by SDS-PAGE; arrows point to the fusion proteins CotB-PA63 (122 kDa), CotB-Domain 4 (75 kDa, CotC-PA63 (75 kDa), and CotC-Domain 4 (28 kDa), respectively; in the last 2 lanes, vegetative cell lysates are fractionated by SDS-PAGE showing PA63 (63 kDa) and Domain 4 (16 kDa) respectively;

Figure 10 shows the results from immune responses after parenteral immunisation wherein a group of mice is immunised (↑) with recombinant *B. subtilis* spores expressing CotB-Domain 4, *rrnO*-PA63 (▲); CotB-Domain 4, *rrnO*-Domain 4 (■); CotC-PA63, *rrnO*-PA63 (△); and CotC-PA63, *rrnO*-Domain 4 (□); sera are tested by ELISA for PA-specific IgG and endpoint titers are

calculated as dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 11 shows the protein sequence listing for *B. anthracis* protective antigen Domain I herein referred to as SEQ ID No. 1;

Figure 12 shows the protein sequence listing for *B. anthracis* protective antigen Domain II herein referred to as SEQ ID No. 2;

Figure 13 shows the protein sequence listing for *B. anthracis* protective antigen Domain III herein referred to as SEQ ID No. 3;

Figure 14 shows the protein sequence listing for *B. anthracis* protective antigen Domain IV herein referred to as SEQ ID No. 4;

Figure 15 shows the DNA sequence listing for promoter (*rrnO*) – RBS (*sspA*) – MCS herein referred to as SEQ ID No. 5;

Figure 16 shows the protein sequence listing for *B. anthracis* protective antigen PA83 herein referred to as SEQ ID No. 6;

Figure 17 shows the protein sequence listing for *B. anthracis* protective antigen PA63 herein referred to as SEQ ID No. 7;

Figure 18 shows the results from immune responses after intraperitoneal immunisation wherein a group of mice is immunised (↑) with recombinant *B. subtilis* expressing the stated fragments of anthrax protective antigen in a vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as

dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 19 shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 18; at day 45 the ELISA and TNA titres of the final sera were measured;

Figure 20 shows the results from immune responses after nasal immunisation wherein a group of mice is immunised (↑)_with recombinant *B. subtilis* expressing the stated fragments of anthrax protective antigen in a vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 21 shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 20; at day 69 the ELISA and TNA titres of the final sera were measured;

Figure 22 shows the results from immune responses after subcutaneous immunisation wherein a group of mice is immunised (↑)_with recombinant *B. subtilis* spores expressing the stated fragments of anthrax protective antigen on the spore coat; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm})

as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 23 shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 22; at day 45 the ELISA and TNA titres of the final sera were measured;

Figure 24 shows the results from immune responses after nasal immunisation wherein a group of mice is immunised (↑)_with recombinant *B. subtilis* spores expressing the stated fragments of anthrax protective antigen on the spore coat; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 25 shows the results from immune responses after oral immunisation wherein a group of mice is immunised (↑)_with recombinant *B. subtilis* expressing the stated fragments of anthrax protective antigen in the vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

Figure 26 shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in

Figure 25; at day 69 the ELISA and TNA titres of the final sera were measured;

Figure 27 shows gene fusions in plasmid pDG364;

Figure 28 shows the DNA sequence listing for Anthrax Protective Antigen PA63 herein referred to as SEQ ID No. 8;

Figure 29 shows the DNA sequence listing for Anthrax Protective Antigen Domain IV herein referred to as SEQ ID No. 9;

Figure 30 shows the DNA sequence listing for Anthrax Protective Antigen Domain DIb23 herein referred to as SEQ ID No. 10;

Figure 31 shows the protein sequence listing for Anthrax Protective Antigen Domain DIb23 herein referred to as SEQ ID No. 11;

Figure 32 shows the DNA sequence listing for B. subtilis CotB protein from residue -263 to residue +825 herein referred to as SEQ ID No. 12;

Figure 33 shows the protein sequence listing for B. subtilis CotB protein from residue -263 to residue +825 herein referred to as SEQ ID No. 13;

Figure 34 shows the DNA sequence listing for B. subtilis CotC protein from residue -179 to residue +198 herein referred to as SEQ ID No. 14;

Figure 35 shows the protein sequence listing for B. subtilis CotC protein from residue -179 to residue+198 herein referred to as SEQ ID No. 15;

Figure 36 shows single constructs of PA83 (full length), PA83, PagD1b23, PA63 and PagD4 with the rrnO promoter in pDL242;

Figure 37 shows the DNA sequence listing for Anthrax PA83 (full length) herein referred to as SEQ ID No. 16; and

Figure 38 shows the DNA sequence listing for Anthrax PA83 herein referred to as SEQ ID No. 17.

Figure 1 illustrates the role of PA. In the first step, PA is secreted from the *B. anthracis* cell. PA is herein referred to as PA83 (full length) and is SEQ ID No. 16. Secretion cleaves the first 29 amino acids of PA83 (full length) to produce the mature PA (PA83). PA83 (735 amino acids) carries 4 domains which are:

Domain 1 (residues 1-250) which is SEQ ID No. 1;
Domain 2 (residues 251-487) which is SEQ ID No. 2;
Domain 3 (488-594) which is SEQ ID No. 3; and
Domain 4 (residues 595-735) which is SEQ ID No. 4.

Domain 4, covering residues 595-735 of the C-terminus of PA is required for receptor binding and monoclonal antibodies specific to this region can block receptor binding.

In the second step of the process illustrated in Figure 1, mature PA83 binds to cell receptor (using domain 4) and is cleaved (at domain 1) by a furin-like protease to free PA20 (subdomain 1a) and expose the EF/LF binding site (in subdomain 1b). The activated PA63s (using domains 2

and 3) heptamerise (Milne et al., 1994), and synchronously bind to EF/LF (up to 3 molecules EF/LF per PA63 heptamer), (Mogridge et al., 2002). The toxin complex is internalised by receptor-mediated endocytosis (Gordon et al., 1988). When the endosome fuses to an acidic compartment, low pH enables the formation of a pore (using domain 2) through the lipid membrane (Blaustein et al., 1989; Koehler and Collier, 1991; Menard et al., 1996; Milne and Collier, 1993) hence the translocation of EF/LF moieties into the cytoplasm. The outcome is then cell death.

The invention will now be illustrated with reference to the following examples which are not intended to limit the scope of the invention claimed.

EXAMPLE 1

The Prrn0-RBS(sspA)-MCS vector was constructed as follows:

rrnO promoter (290 bp) was amplifed from *B.subtilis* chromosome (PY79) with forward (F) primer containing a BglII site, and reverse (R) primer containing a XbaI site.

sspA ribosome binding site (RBS) of *B.subtilis* (20 bp) was synthesised by annealing 2 oligonucleotides so that the double stranded DNA contains 2 cohesive ends, XbaI at 5' and NcoI at 3'.

These 2 fragments were cloned into pET28b restricted with BglII and NcoI.

rrnO promoter, *sspA* RBS, and MCS from pET28b were amplified with 2 primers containing PvuII site. The PCR product (Prm-RBS-MCS) was restricted with PvuII to create a blunt-ended DNA.

Plasmids pDG364 and pDG1664 were restricted with EcoRI and BamHI, and blunt-ended by DNA polymerase I (Klenow) before ligated to the above (Prrn0-RBS-MCS) DNA fragment.

The MCS are as follows (from the start codon ATG): NdeI, NheI, BamHI, EcoRI, SacI, SalI, HindIII, NotI, XhoI.

EXAMPLE 2

Construction of Recombinant *B. subtilis* Strains

The non-pathogenic spore-forming bacterium *Bacillus subtilis* was engineered to express different domains of the protective antigen (PA) from *Bacillus anthracis*.

The domains chosen were: mature secreted PA83 (735 aa, 82.7 kDa), functional PA63 (568 aa, 63.5 kDa), and Domain 4 of PA (141 aa, 16.1 kDa).

The ways in which these antigens were displayed are: in-frame fusion with the *B. subtilis* spore coat (*cotB* and *cotC*) proteins for spore coat expression, or under the constitutive ribosomal RNA promoter (*rrnO*) for vegetative cell expression. Briefly, the fusion recombinant DNA (*cotB/cotC* with their promoters – antigens, or *rrnO* promoter – antigens) are introduced into *B. subtilis* chromosome by double-crossover integration at the *amyE* or *thrC* loci. The constructs (recombinant *B.*

subtilis expressing heterologous antigens) are selected by means of antibiotic markers (Figure 4 and 5).

The single constructs (spores expressing one antigen) are shown in Figures 6 and 7:

1. CotB-PA63
2. CotB-Domain 4
3. CotC-PA63
4. CotC-Domain 4

The following constructs are shown in Figure 8:

5. rrnO-PA83
6. rrnO-PA63
7. rrnO-Domain 4

The following double constructs (spores expressing two antigens) were also used:

9. CotB-Domain 4, rrnO-PA83 (2 and 5)
10. CotB-Domain 4, rrnO-PA63 (2 and 6)
11. CotB-Domain 4, rrnO-Domain 4 (2 and 7)
12. CotB-Domain 4, rrnO-sLTB (2 and 8)
13. CotC-PA63, rrnO-PA83 (3 and 5)
14. CotC-PA63, rrnO-PA63 (3 and 6)
15. CotC-PA63, rrnO-Domain 4 (3 and 7)
16. CotC-PA63, rrnO-sLTB (3 and 8)

Expression of PA on spore coat or in vegetative cells is checked by Western blots (Figure 9) and confocal immunofluorescent microscopy.

EXAMPLE 3

Evaluation of Immune Responses

With the constructs of Example 2, groups of 8 A/J inbred mice were immunised by different routes: intra-peritoneal (i.p.), oral, and intra-nasal (i.n.).

Parenteral immunisation (Figure 10)

Intra-peritoneal injection utilised 3 doses of $1x10^9$ spores on days 0, 20 and 40. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 55. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 10). Control groups were non-immunised (naïve), or immunised with non-recombinant spores, or with purified PA protein. This study reveals constructs that are most immunogenic (titers > 2,000) and pilots the mucosal immunisations.

Mucosal immunisations

Mucosal immunisations utilise 2 routes. Mice were dosed orally with $1x10^{10}$ spores, or intra-nasally with $1x10^9$ spores on days 0, 20 and 40. Control groups are included as in the i.p. route. The immune responses were assessed by various methods. Anti-PA serum IgG and its subclass (IgG1, IgG2a, IgG2b, IgG3) titres were determined by ELISA, so were fecal secreted IgA (for oral) and saliva IgA (for i.n.) titres on day 55. The results show the type of immune responses to PA expressed in *B. subtilis* mucosally administered to mice. The spore-specific responses were examined to further understand the nature of immunogenicity when using *B. subtilis* spores as mucosal delivery vehicles of heterologous antigens.

Preliminary results show sero-conversion by the nasal and oral routes.

EXAMPLE 4

Construction of further Recombinant *B. subtilis* Strains

The non-pathogenic spore-forming bacterium *Bacillus subtilis* was engineered to express different domains of the protective antigen (PA) from *Bacillus anthracis*.

The domains chosen were: PA83 (full length, 764aa), mature secreted PA83 (735 aa, 82.7 kDa), PagD1b23 (47kDa, domain D1b23), functional PA63 (568 aa, 63.5 kDa), Domain 4 of PA (also herein referred to as PagD4, 141 aa, 16.1 kDa).

The ways in which these antigens were displayed are: in-frame fusion with the *B. subtilis* spore coat (*cotB* and *cotC*) proteins for spore coat expression, or under the constitutive ribosomal RNA promoter (*rrnO*) for vegetative cell expression. Briefly, the fusion recombinant DNA (*cotB/cotC* with their promoters – antigens, or *rrnO* promoter – antigens) are introduced into *B. subtilis* chromosome by double-crossover integration at the *amyE* or *thrC* loci. The constructs (recombinant *B. subtilis* expressing heterologous antigens) are selected by means of antibiotic markers (Figure 4 and 5).

The following constructs in plasmid pDG364 are shown in Figure 27:

1. CotB-PA63
2. CotB-PagD4
3. CotC-PagD4

The following constructs in plasmid pDL242 are shown in Figure 37:

4. rrnO-PA83 (full length)
5. rrnO-PA83
6. rrnO-PagD1b23

7. rrnO-PA63
8. rrnO-PagD4

The spores transformed by construct 4 of this Example are referred to as PA83 sec. This is because in the vegetative cell state the PA83 antigen is secreted from the cell. The PA83 (full length) protein which is expressed by the construct includes a 29 amino acid leader sequence which enables secretion. This leader sequence is chopped off the protein as it is translocated across a cell membrane and released from the cell. Thus the secreted polypeptide is PA83, not PA83 (full length).

The spores transformed by construct 5 and 7 of this Example are referred to as PA83 intra and PA63 intra because the antigen is not secreted from the cell; instead it is only available intra-cellularly.

The following spores were also used:

9. CotC-PagD4	Domain 4 of PA fused to the CotC spore coat protein
10. CotB-PagD4	Domain 4 of PA fused to CotB spore coat protein
11. CotB-PagD1b23	Domain D1b23 fused to CotB spore coat protein

For the CotB/C constructs, the initial fusion of gene sequences are made in E. coli and then subcloned into pDG364 (using a MCS). Next, pDG364 is linearised and introduced into B. subtilis cells by DNA mediated transformation as shown in Figure 5.

For rrnO constructs that permit vegetative gene expression, the relevant gene sequence is cloned into the MCS (multiple cloning site) of pDL242 (Figure 2) to allow fusion to the rbs of the sspA gene under the control of

the PrrnO promoter. It is noted that rrnO is a gene only expressed in vegetative cells, that is, only in the germinating/germinated spore. Coat proteins (ie, CotC and/or CotB are surface exposed proteins on the spore.

The pDL242 recombinant plasmid is then linearised and introduced into B. subtilis cells by DNA mediated transformation as shown in Figure 4.

EXAMPLE 5

Evaluation of Immune Responses

With the constructs of Example 4, groups of 6 Balb/C inbred mice were immunised by different routes: intra-peritoneal (i.p.), subcutaneous, intra-nasal (i.n.) and oral.

Intraperitoneal immunisation (Figures 18 and 19)

Intra-peritoneal injection utilised 3 doses of $1x10^9$ spores on days 0, 16 and 29. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 45. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 18) and using the TNA assay of Example 7 (Figure 19). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with a 100 μl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Human anthrax vaccine is a cell-free extract of B. anthracis culture medium (Sterne strain). The medium extract contains unknown amount of PA (mainly) and LF/EF (fraction) and other secreted proteins of the B.

anthracis strain. The extract is absorbed with Alum (aluminium hydroxide/phosphate).

Nasal immunisation (Figures 20 and 21)

Nasal injection utilised 51 doses of $2x10^9$ spores on each day from day 0 to day 50. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 20) and using the TNA assay of Example 7 (Figure 21). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with a 20 μl/dose (which is one twenty fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Subcutaneous immunisation (Figures 22 and 23)

Subcutaneous injection utilised 3 doses of $1x10^9$ spores on days 0, 16 and 29. The spores had been transformed with spore coat expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 45. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 22) and using the TNA assay of Example 7 (Figure 23). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with 100 μl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Nasal immunisation (Figure 24)

Nasal injection utilised 51 doses of $2x10^9$ spores on each day from day 0 to day 50. The spores had been transformed with spore coat expression

constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 24). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with 100 μl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Oral immunisation (Figures 25 and 26)

Oral injection utilised 7 doses of $1x10^9$ spores on days 1, 2, 3, 21, 22, 35 and 36. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 25) and using the TNA assay of Example 7 (Figure 26). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with a 20 μl/dose (which is one twenty fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

EXAMPLE 6

The following methodology was used in the anti-PA ELISA assay.

Plates were coated with 50 μl/well of purified protective antigen (1 μg/ml in PBS) and left at room temperature overnight. After blocking with 2% BSA in PBS for 1.5 h at 37°C serum samples were applied using a 2-fold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1M Tris-HCl, pH 7.4; 3% (w/v) NaCl; 2% (w/v) BSA; 10% (v/v) fetal bovine serum (Sigma); 0.1% (v/v) Triton-X-100; 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/40 diluted

pre-immune serum), a positive control (serum from mice immunised parentally with protective antigen). Plates were incubated for 1 h at 37°C before addition of anti-mouse HRP conjugate (Sigma). Plates were incubated for a further 1 h at 37°C then developed using the substrate TMB (3, 3', 5, 5'-tetramethyl-benzidine; Sigma). Reactions were stopped using 2M H_2S0_4. Dilution curves were drawn for each sample and endpoint titres calculated as the dilution producing the same optical density as the 1/40 dilution of a pooled pre-immune serum.

EXAMPLE 7

The following methodology was used in the Toxin Neutralisation Assay (TNA).

The murine macrophage-like cell line RAW264.7 (obtained from the European Collection of Animal Cell Cultures [ECACC]) was cultured as monolayers in DMEM medium (Sigma) supplemented with 10% (v/v) fecal bovine serum, 50 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, in an atmosphere of 90% humidity containing 5% CO_2 at 37°C. One day before use, the cells were detached by gentle scraping and seeded into 96-multiwell disposable plates in the same medium with 0.1 mM HEPES at a density of approximately 1 x 10^5 cells per well. Test sera were serially diluted in DMEM/HEPES medium, and mixed with anthrax lethal toxin (0.1 μg/ml LF, 0.08 μg/ml PA [Quadratic Diagnostics]) with volume ratio 1:1 in a separate 96-well plate. After 1 h incubation at 37°C, corresponding wells were transferred to the macrophage culture plate. After 3 h incubation at 37°C, surviving macrophages were measured by addition of WST-1 reagent (Roche) and further incubation for 4 h at 37°C.The absorbance was read at 450 nm wavelength, and results were scored against positive (medium only) and negative (toxin only) controls.

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CLAIMS

1. A non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen.

2. A spore as defined in claim 1 which is a Bacillus species spore; preferably a spore from one or more of the following organisms: *Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis; Bacillus mycoides; Bacillus pasteurii; Bacillus sphaericus; Bacillus aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus freudenreichi; Bacillus macroides; Bacillus similibadius; Bacillus thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis; Bacillus bengalicus; Bacillus flexus;* and/or *Bacillus licheniformis*; more preferably a spore from *Bacillus subtilis*.

3. A spore as defined in claim 1 or claim 2 which comprises an antigenic fragment of anthrax protective antigen in the form of a protein attached to the proteinaceous coat of the spore.

4. A spore as defined in any one of the preceding claims which comprises an antigenic fragment of anthrax protective antigen encoded in the form of DNA which is adapted to be expressed when the spore germinates.

5. A spore as defined in any one of the preceding claims wherein the antigenic fragment is one or more of:

PA83 which is SEQ ID No. 6;

PA63 which is SEQ ID No. 7;

Domain 1 of the protective antigen which is SEQ ID No. 1;

Domain 2 of the protective antigen which is SEQ ID No. 2;

Domain 3 of the protective antigen which is SEQ ID No. 3;

Domain 4 (residues 595-735) of the protective antigen which is SEQ ID No. 4; and

Domain D1b23 which is SEQ ID No. 10.

6. A spore substantially as hereinbefore described.

7. A spore as defined in any one of the preceding claims which is for use as an anthrax vaccine.

8. A pharmaceutical composition comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in association with a pharmaceutically acceptable carrier and/or excipient.

9. A composition as defined in claim 8 wherein the spore is a Bacillus species spore; preferably a spore from one or more of the following organisms: *Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis; Bacillus mycoides; Bacillus pasteurii; Bacillus sphaericus; Bacillus aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus freudenreichi; Bacillus macroides; Bacillus similibadius; Bacillus thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis; Bacillus bengalicus; Bacillus flexus;* and/or *Bacillus licheniformis*; more preferably a spore from *Bacillus subtilis*.

10. A composition as defined in claim 8 or claim 9 wherein the spore comprises at least an antigenic fragment of anthrax protective antigen in the form of a protein attached to the proteinaceous coat of the spore.

11. A composition as defined in any one of claims 8 to 10 wherein the spore comprises at least an antigenic fragment of anthrax protective

antigen encoded in the form of DNA which is adapted to be expressed when the spore germinates.

12. A composition as defined in any one of claims 8 to 11 which comprises an antigenic fragment of anthrax protective antigen, preferably the fragment is one or more of:

PA83 which is SEQ ID No. 6;

PA63 which is SEQ ID No. 7;

Domain 1 of the protective antigen which is SEQ ID No. 1;

Domain 2 of the protective antigen which is SEQ ID No. 2;

Domain 3 of the protective antigen which is SEQ ID No. 3;

Domain 4 (residues 595-735) of the protective antigen which is SEQ ID No. 4; and

Domain D1b23 which is SEQ ID No. 10.

13. A composition as defined in any one of claims 8 to 12 which further comprises an adjuvant which potentiates an antigen-specific immune response.

14. A composition as defined in any one of claims 8 to 13 wherein the spore is substantially as hereinbefore described.

15. A composition as defined in any one of claims 8 to 14 for use as an anthrax vaccine.

16. Use of a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in the manufacture of a vaccine for anthrax for non-parenteral administration, preferably for nasal and/or oral administration.

17. Use according to claim 16 wherein the spore is as defined in any one of claims 9 to 12.

18. Use of a composition as defined in any one of claims 8 to 15 in the manufacture of a vaccine for anthrax.

19. Use of a spore as defined in any one of claims 1 to 7 in the manufacture of a vaccine for anthrax.

20. Use as defined in claim 18 or claim 19 wherein the vaccine is for non-parenteral administration, preferably for nasal and/or oral administration.

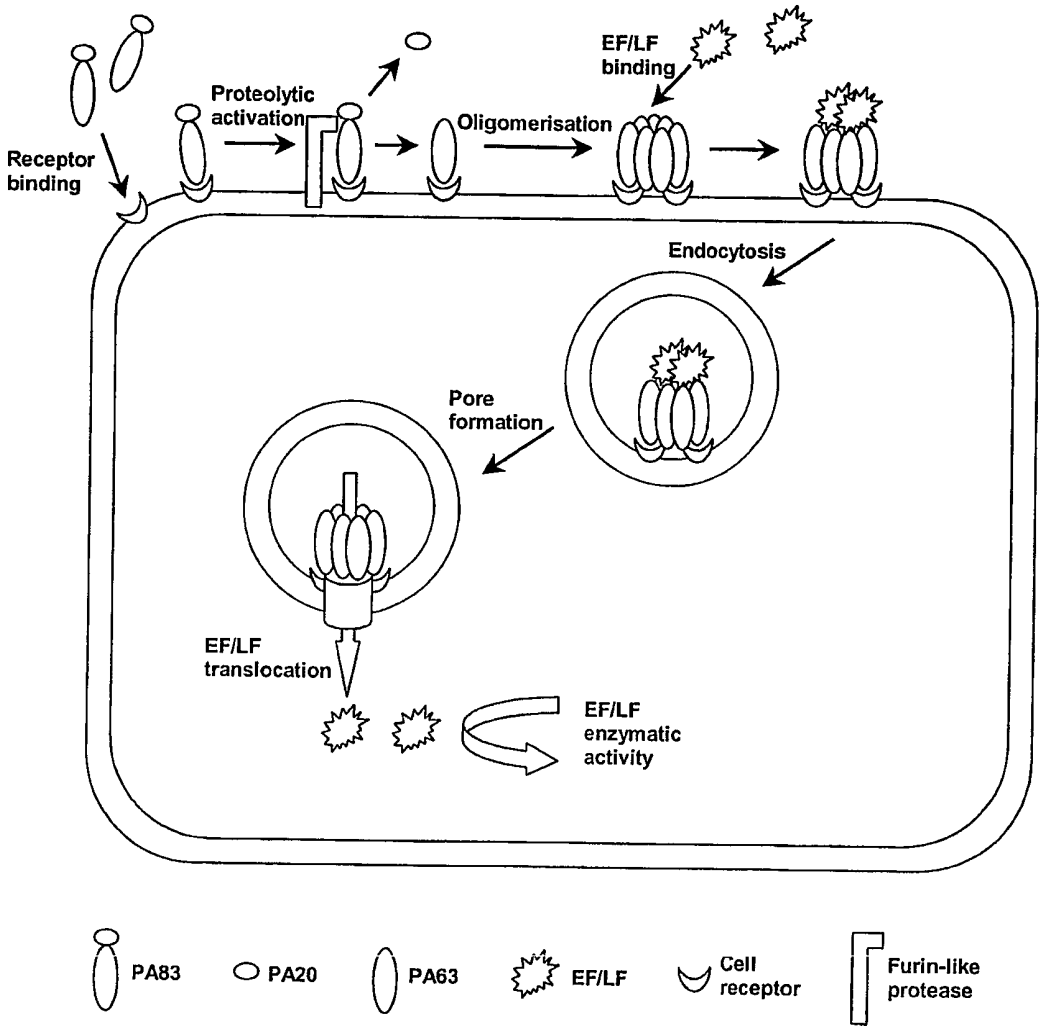
21. A method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises non-parenteral administration, preferably nasal or oral administration of an effective amount of a vaccine comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen.

22. A method as defined in claim 21 wherein the spore is as defined in any one of claims 9 to 12.

23. A method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises administration of an effective amount of a vaccine comprising a spore as defined in any one of claims 1 to 7 or of a composition as defined in any one of claims 8 to 15.

24. A method as defined in claim 23 wherein the vaccine is for non-parenteral administration, preferably for nasal and/or oral administration.

Figure 1


Receptor binding
Proteolytic activation
Oligomerisation
EF/LF binding
Endocytosis
Pore formation
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PA83
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EF/LF
Cell receptor
Furin-like protease

Figure 2

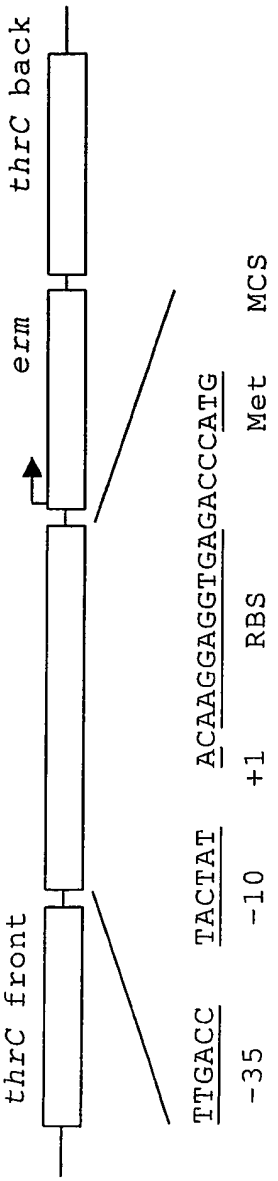

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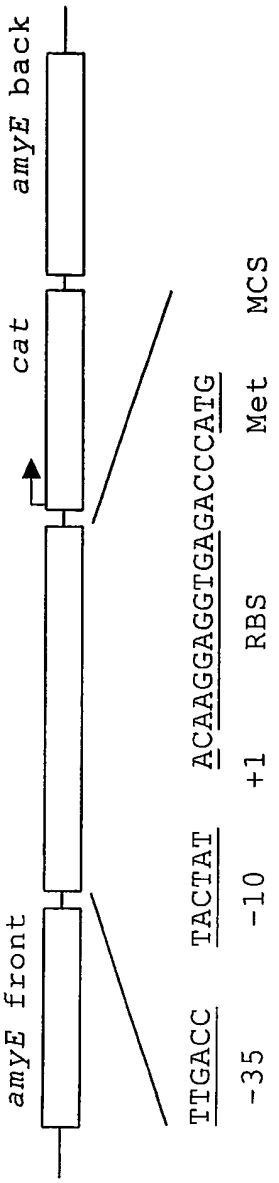
Figure 3



DL243

Figure 4

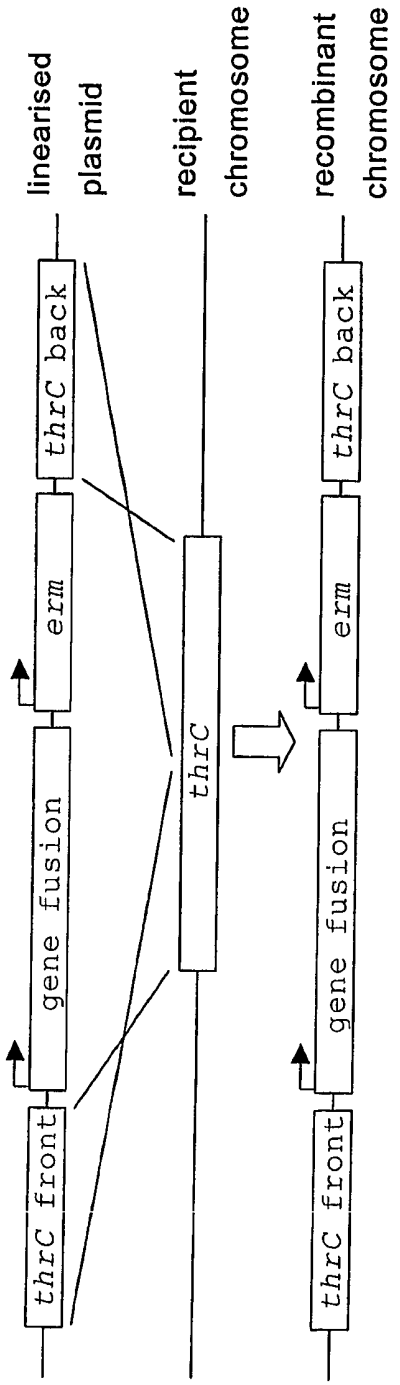

linearised plasmid
recipient chromosome
recombinant chromosome
thrC back
erm
gene fusion
thrC front
thrC

Figure 5

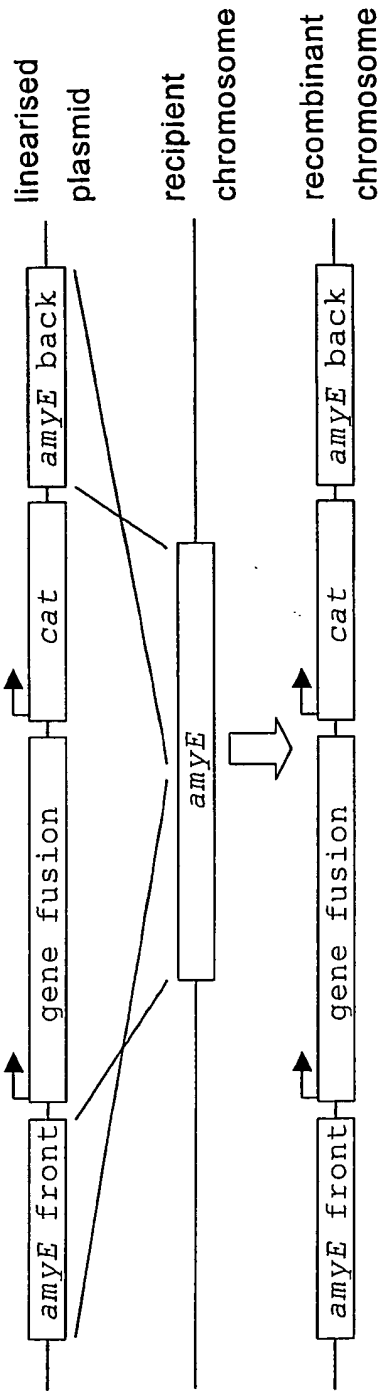

linearised plasmid
recipient chromosome
recombinant chromosome
amyE front
gene fusion
cat
amyE back
amyE
amyE front
gene fusion
cat
amyE back

Figure 6

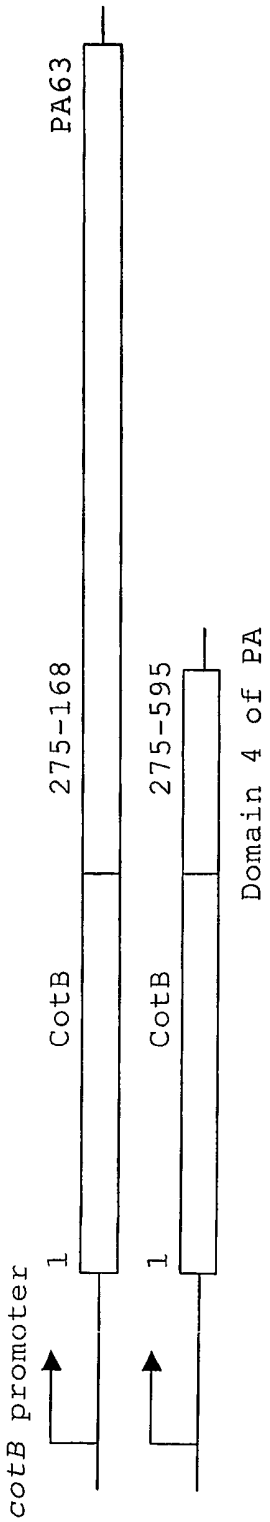


Figure 7

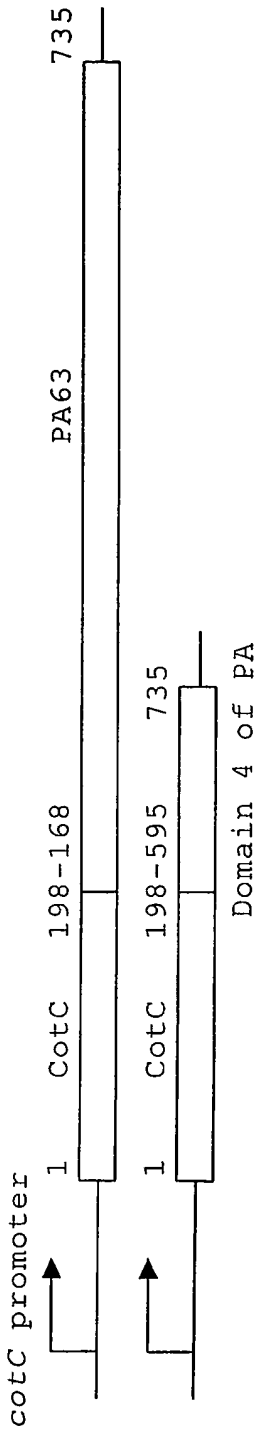

cotC promoter
1
CotC
198-168
PA63
735
1
CotC
198-595
735
Domain 4 of PA

Figure 8

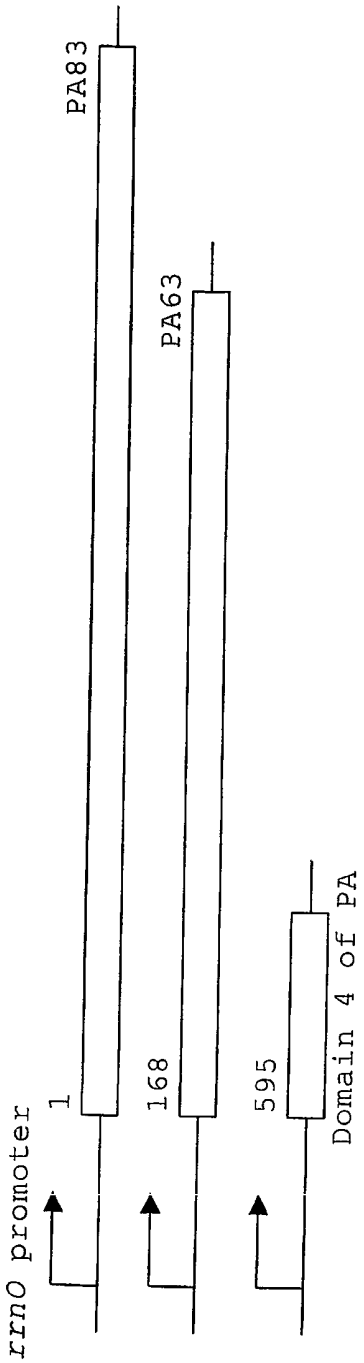
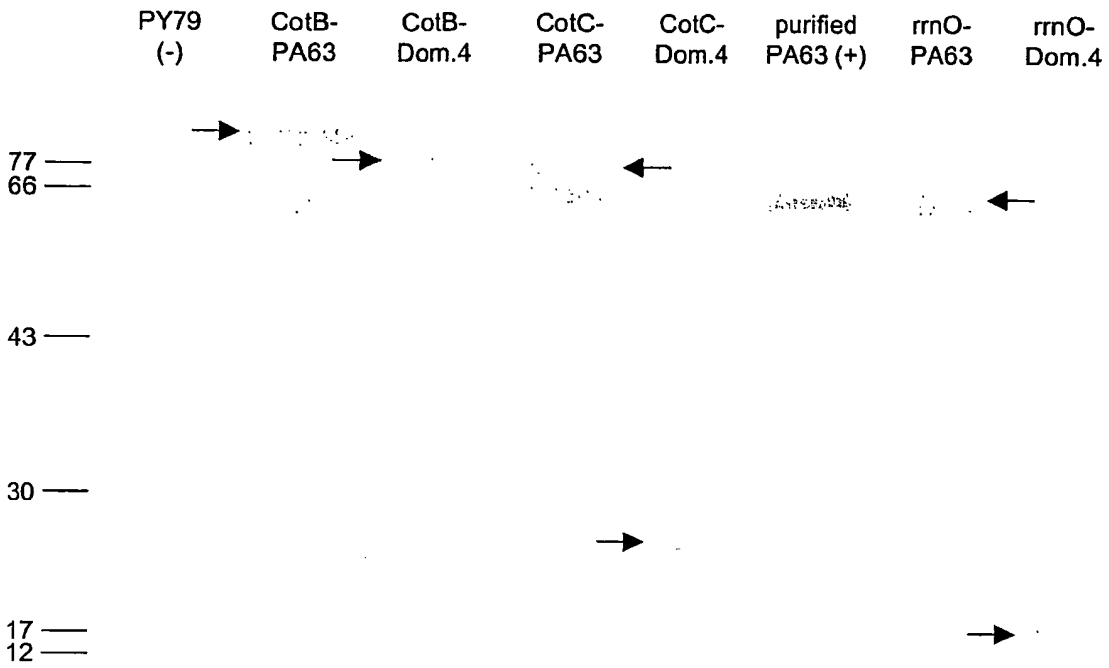


Figure 9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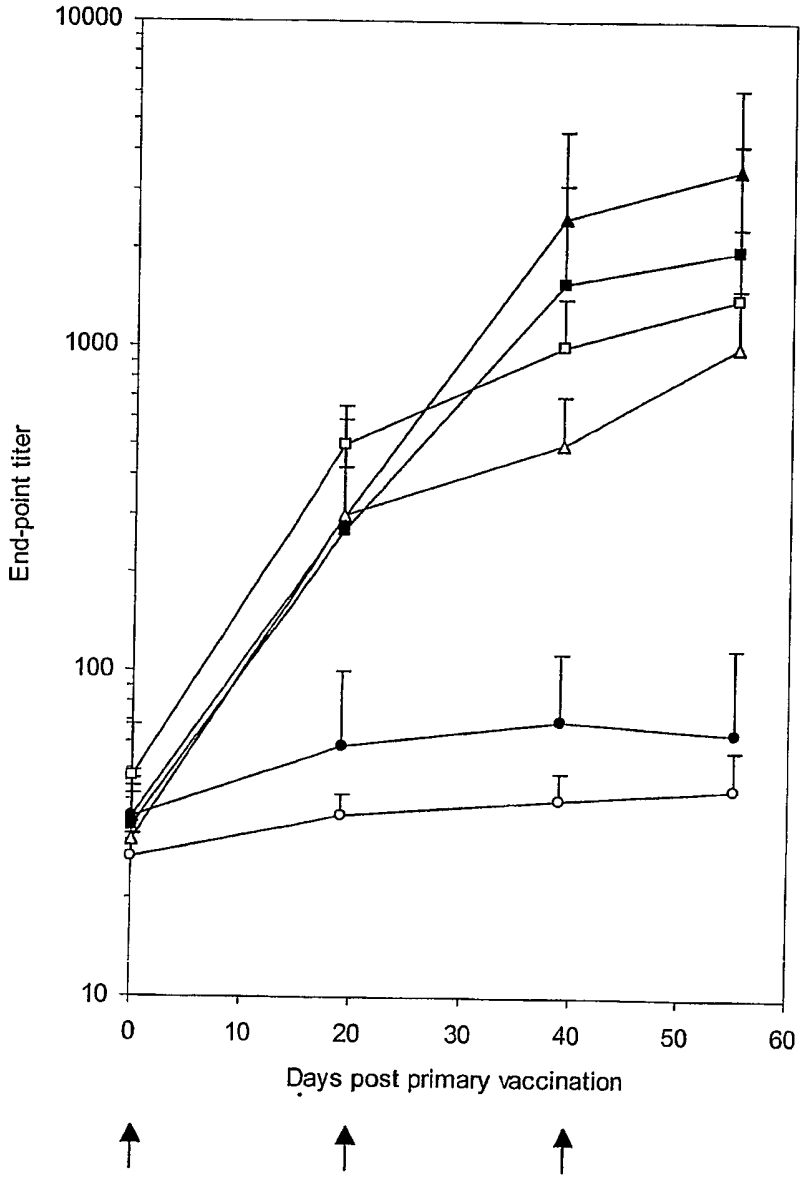
CotB-PA63
CotB-Dom.4
CotC-PA63
CotC-Dom.4
purified PA63 (+)
rrnO-PA63
rrnO-Dom.4
77
66
43
30
17
12

Figure 10


10000
1000
100
10
End-point titer
0
10
20
30
40
50
60
Days post primary vaccination

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QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY    120
QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN    180
DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT    240
GRIDKNVSPE                                                           250
```

Figure 11

```
ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSQTRTIS KNTSTSRTHT SEVHGNAEVH     60
ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL NANIRYVNTG    120
TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA LNAQDDFSST    180
PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV LPQIQET       237
```

Figure 12

```
TARIIFNGKD LNLVERRIAA VNPSDPLETT KPDMTLKEAL KIAFGFNEPN GNLQYQGKDI     60
TEFDFNFDQQ TSQNIKNQLA ELNVTNIYTV LDKIKLNAKM NILIRDK                  107
```

Figure 13

```
RFHYDRNNIA VGADESVVKE AHREVINSST EGLLLNIDKD IRKILSGYIV EIEDTEGLKE     60
VINDRYDMLN ISSLRQDGKT FIDFKKYNDK LPLYISNPNY KVNVYAVTKE NTIINPSENG    120
DTSTNGIKKI LIFSKKGYEI G                                              141
```

Figure 14

```
gatcgacgat atctgcgatg cgtccggcgt agaggatcga gatctgcatg accattatga      60
ctagtaaaaa ctttttcaaa aaagtattga cctagttaac taaaaatgtt actattaagt     120
agtcgctttg agagaagcac acaaagttct ttgaaaacta aacaagacaa aacgtacctg     180
ttaattcatt tttataaatc gcacagcgat gtgcgtagtc agtcaaacta gggcctgcac     240
gacgcaggtc acacaggtgt cgccgcagga tgcggtgaac ttaacctgtt ctagaacaag     300
gaggtgagac ccatgggcag cagccatcat catcatcatc acagcagcgg cctggtgccg     360
cgcggcagcc atatggctag catgactggt ggacagcaaa tgggtcggga tccgaattcg     420
agctccgtcg acaagcttgc ggccgcactc gagcaccacc accaccacca ctgagatccg     480
gctgcc                                                                486
```

Figure 15

```
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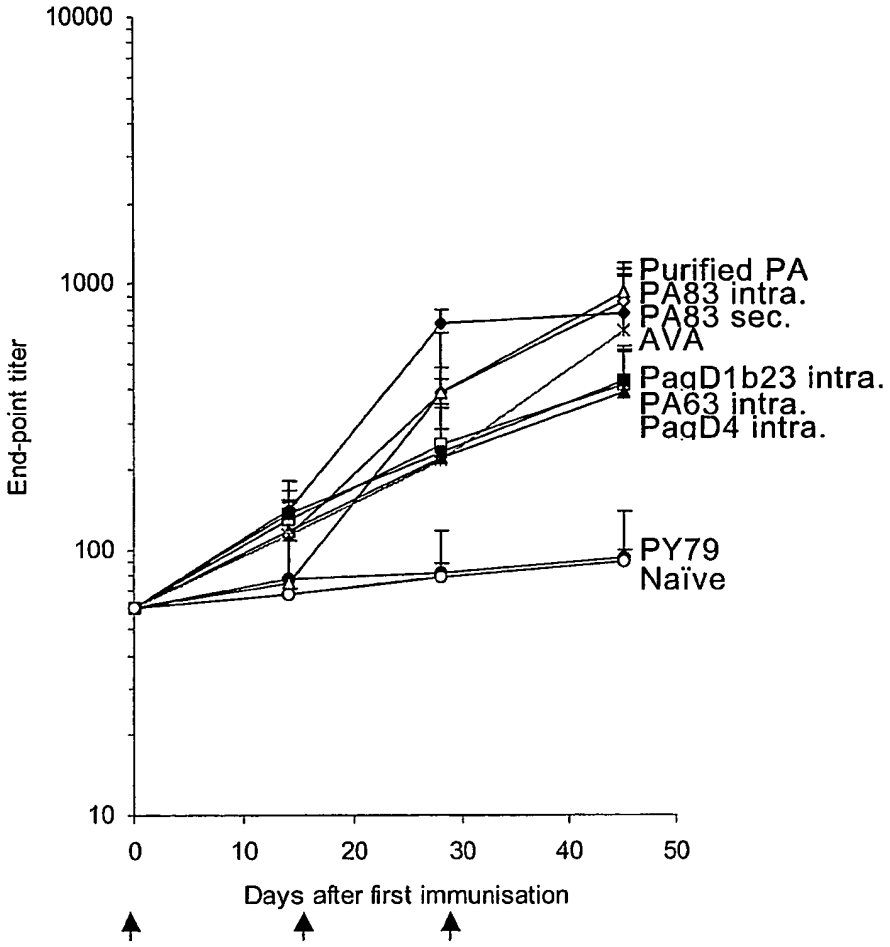
QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS SNSRKKRSTS AGPTVPDRDN     180
DGIPDSLEVE GYTVDVKNKR TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT     240
GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSQTRTIS KNTSTSRTHT     300
SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL     360
NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ LSQILAPNNY YPSKNLAPIA     420
LNAQDDFSST PITMNYNQFL ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV     480
LPQIQETTAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL     540
QYQGKDITEF DFNFDQQTSQ NIKNQLAELN VTNIYTVLDK IKLNAKMNIL IRDKRFHYDR     600
NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS GYIVEIEDTE GLKEVINDRY     660
DMLNISSLRQ DGKTFIDFKK YNDKLPLYIS NPNYKVNVYA VTKENTIINP SENGDTSTNG     720
IKKILIFSKK GYEIG                                                      735
```

Figure 16

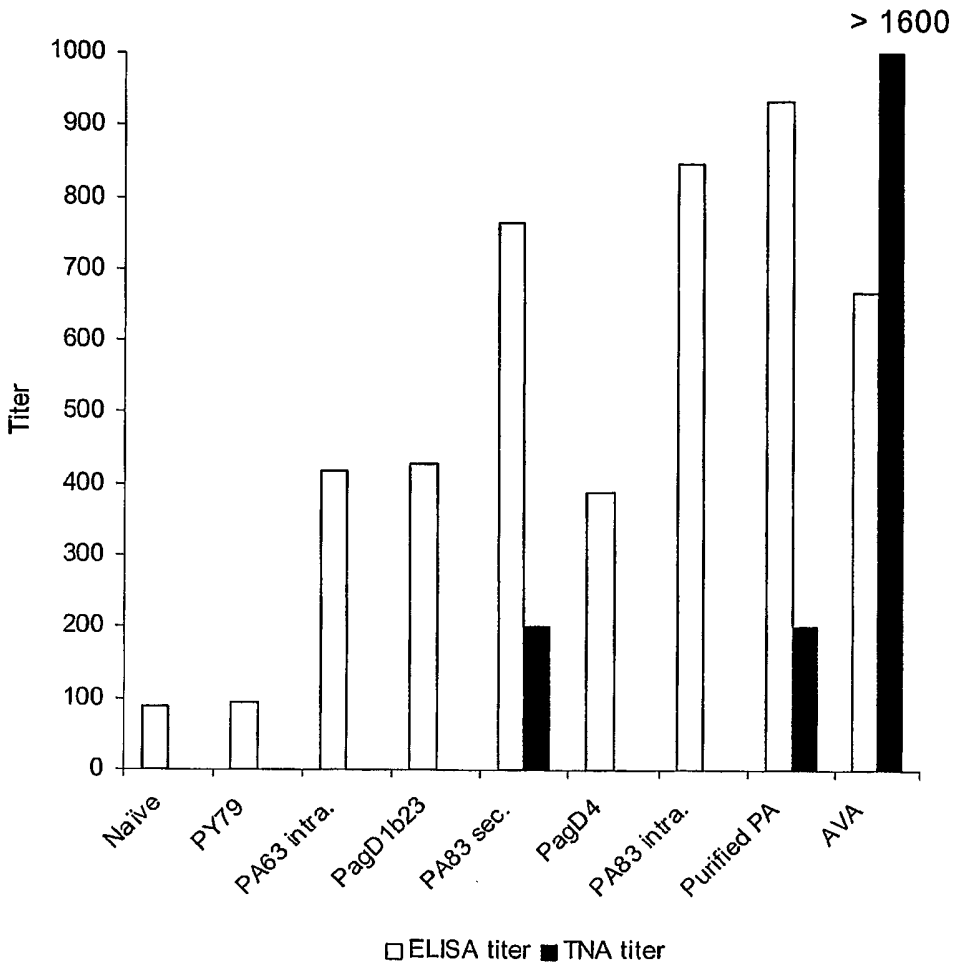
```
STSAGPTVPD RDNDGIPDSL EVEGYTVDVK NKRTFLSPWI SNIHEKKGLT KYKSSPEKWS      60
TASDPYSDFE KVTGRIDKNV SPEARHPLVA AYPIVHVDME NIILSKNEDQ STQNTDSQTR     120
TISKNTSTSR THTSEVHGNA EVHASFFDIG GSVSAGFSNS NSSTVAIDHS LSLAGERTWA     180
ETMGLNTADT ARLNANIRYV NTGTAPIYNV LPTTSLVLGK NQTLATIKAK ENQLSQILAP     240
NNYYPSKNLA PIALNAQDDF SSTPITMNYN QFLELEKTKQ LRLDTDQVYG NIATYNFENG     300
RVRVDTGSNW SEVLPQIQET TARIIFNGKD LNLVERRIAA VNPSDPLETT KPDMTLKEAL     360
KIAFGFNEPN GNLQYQGKDI TEFDFNFDQQ TSQNIKNQLA ELNVTNIYTV LDKIKLNAKM     420
NILIRDKRFH YDRNNIAVGA DESVVKEAHR EVINSSTEGL LLNIDKDIRK ILSGYIVEIE     480
DTEGLKEVIN DRYDMLNISS LRQDGKTFID FKKYNDKLPL YISNPNYKVN VYAVTKENTI     540
INPSENGDTS TNGIKKILIF SKKGYEIG                                        568
```

Figure 17

Figure 18

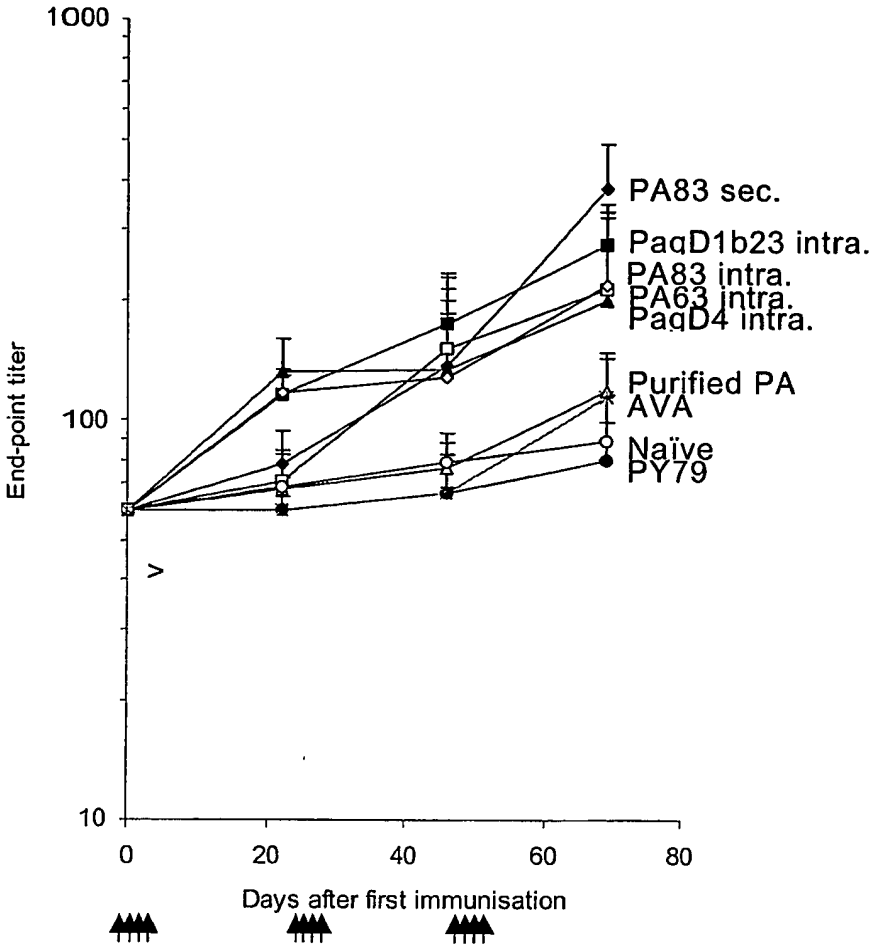


ELISA titers *vs* time for Intraperitoneal Immunisation with vegetative cell expression constructs



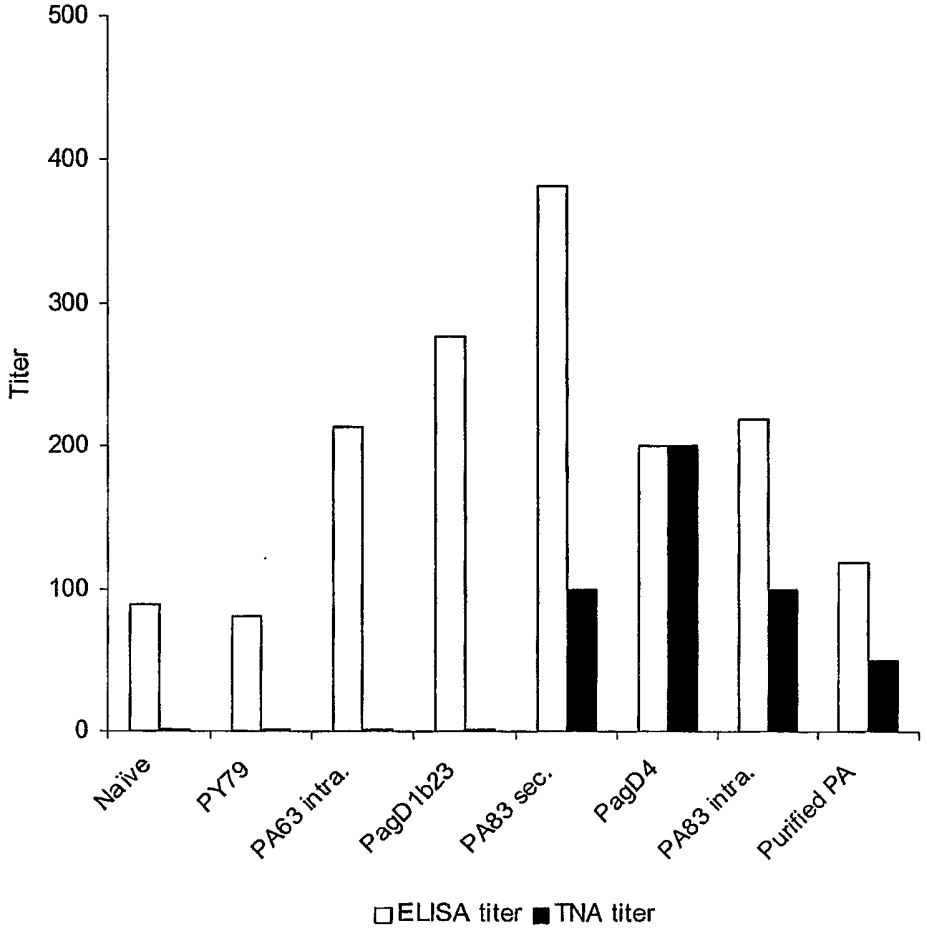
ELISA and TNA titers of final sera (Day 45) for Intraperitoneal Immunisation with vegetative cell expression constructs

Figure 19



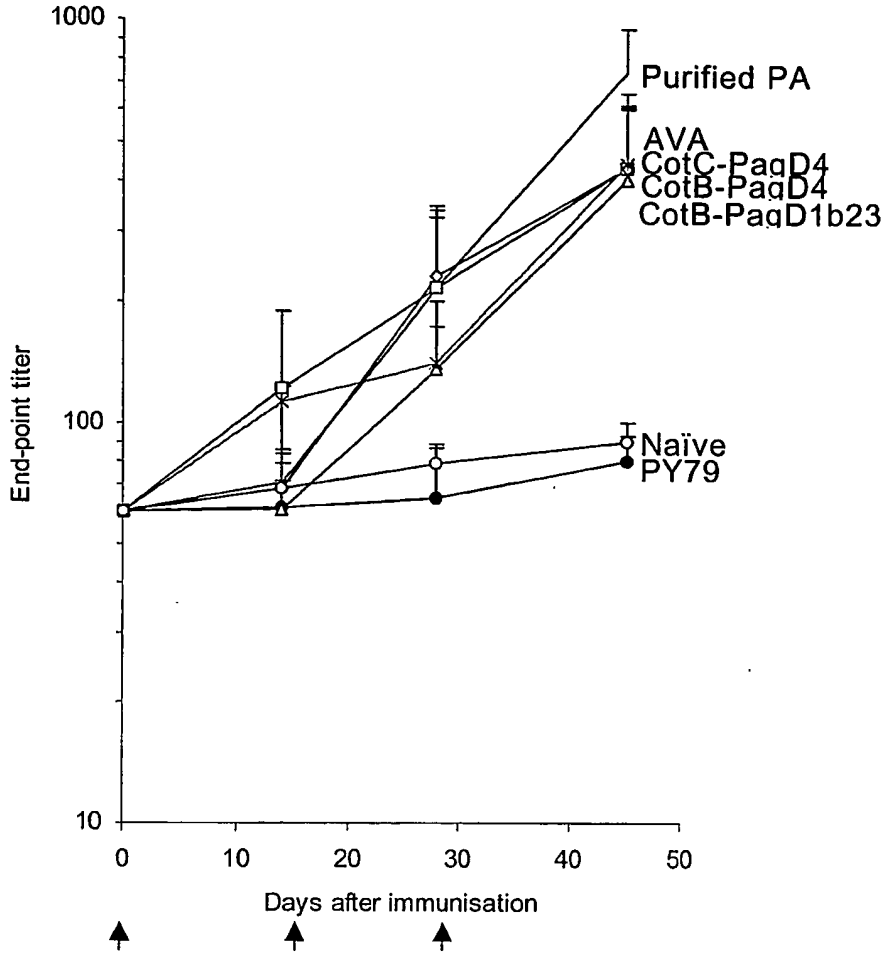
ELISA titers *vs* time for Nasal Immunisation with vegetative cell expression constructs

Figure 20



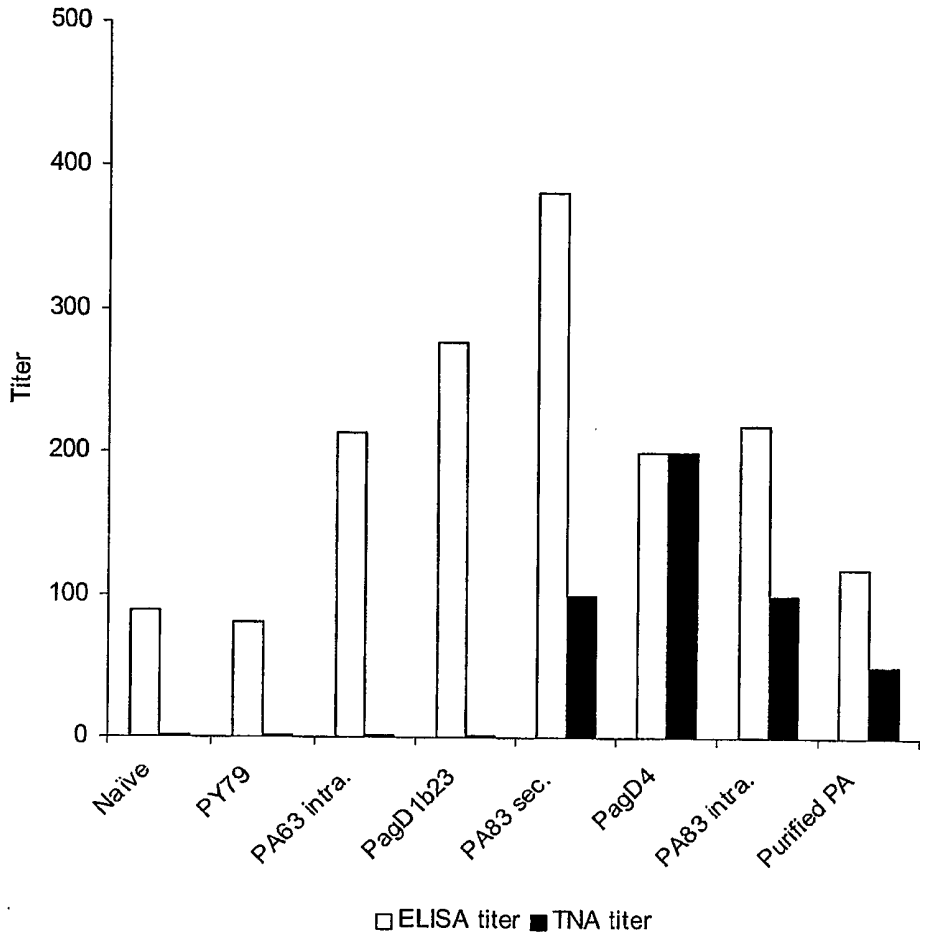
ELISA and TNA titers of final sera (Day 69) for Nasal Immunisation with vegetative cell expression constructs

Figure 21



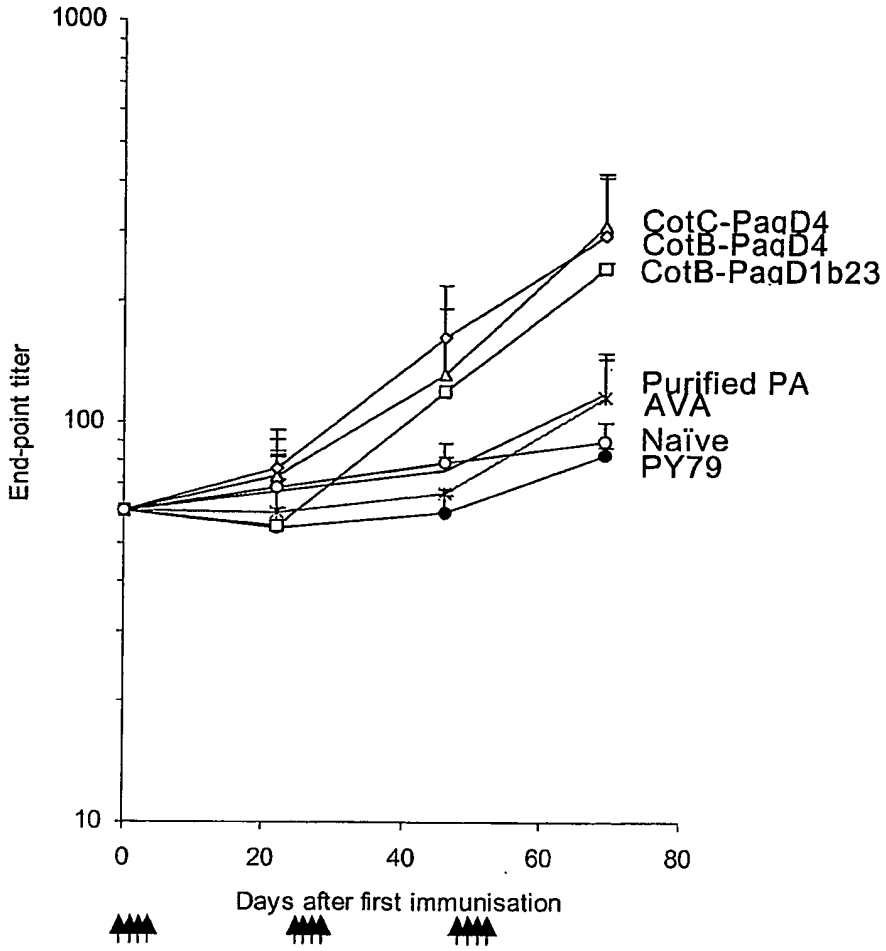
ELISA titers *vs* time for Subcutaneous Immunisation with spore coat expression constructs

Figure 22



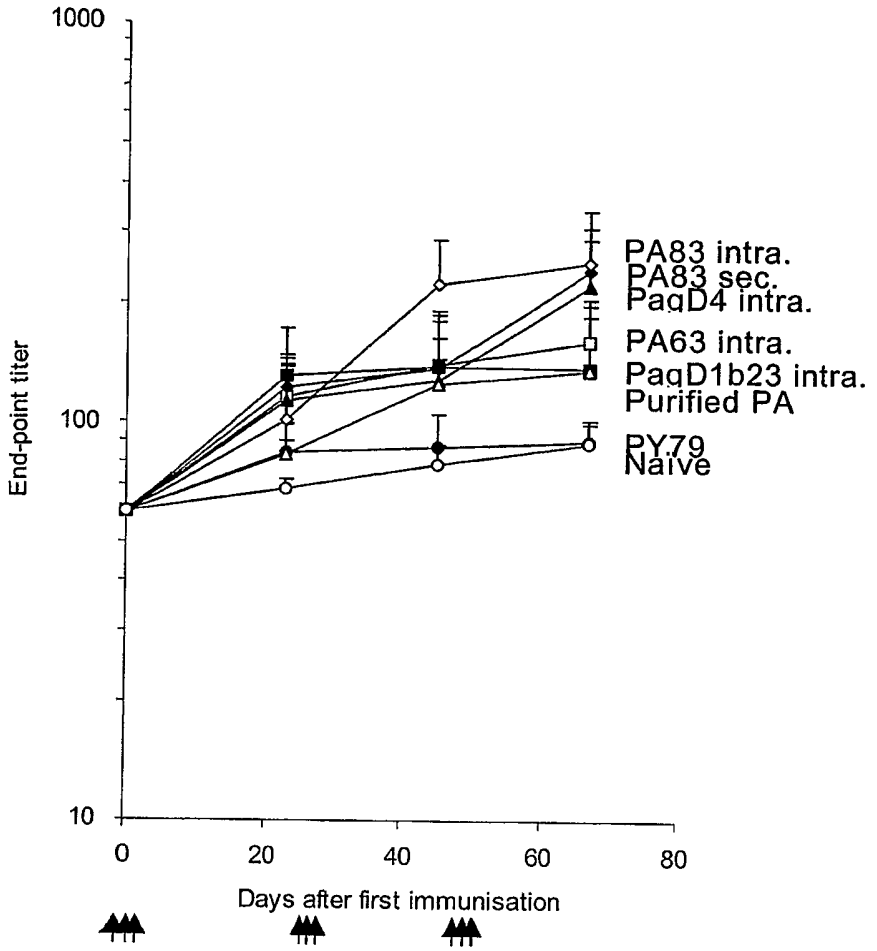
ELISA and TNA titers of final sera (Day 45) for Subcutaneous Immunisation with spore coat expression constructs

Figure 23



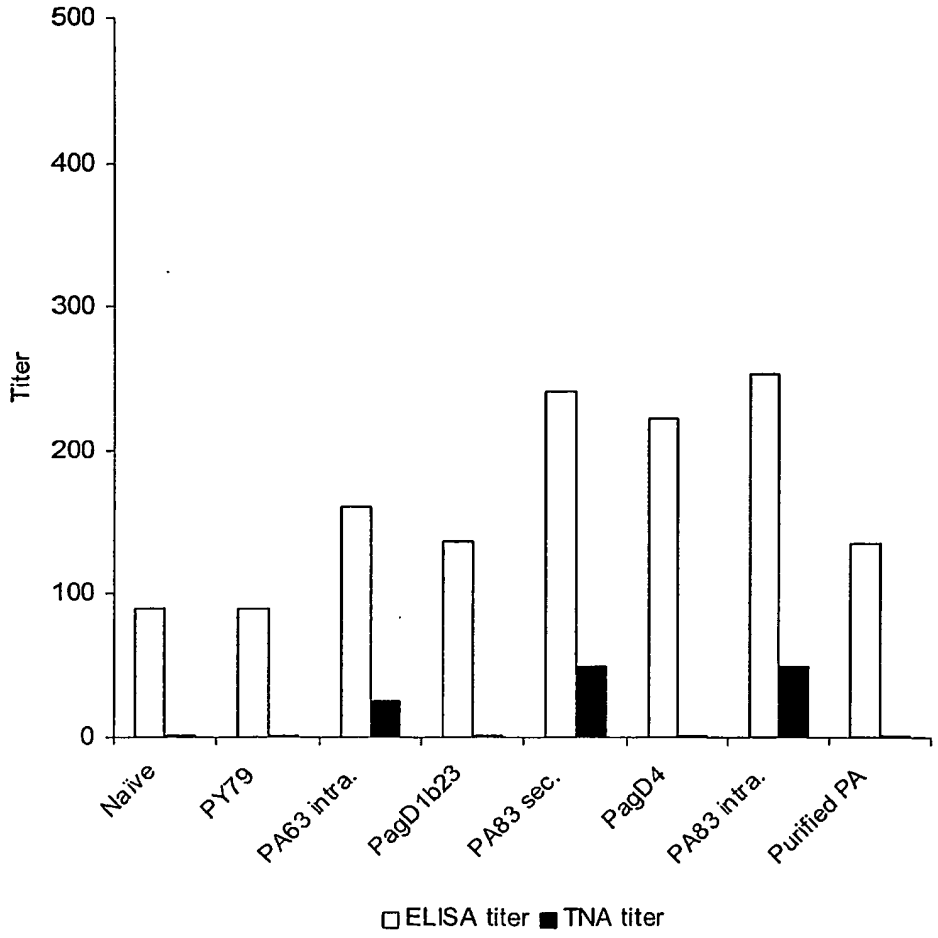
ELISA titers *vs* time for Nasal Immunisation with spore coat expression constructs

Figure 24



ELISA titers *vs* time for Oral Immunisation with vegetative cell expression constructs

Figure 25



ELISA and TNA titers of final sera (Day 69) for Oral Immunisation with vegetative cell expression constructs

Figure 26

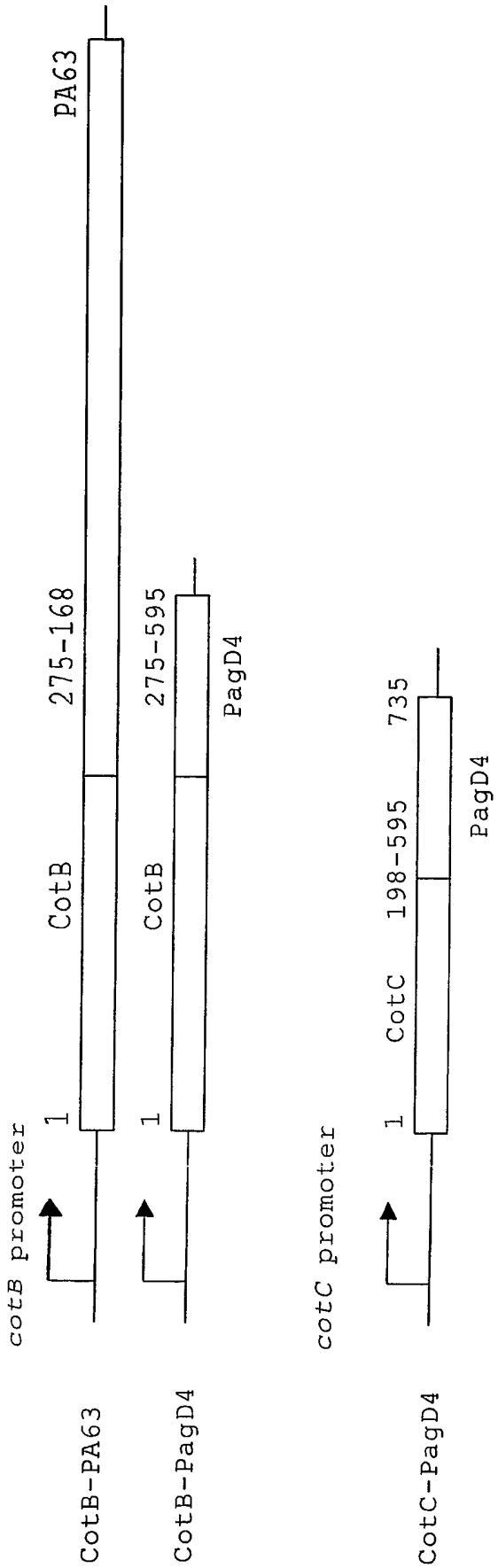

cotB promoter
1
CotB
275-168
PA63
CotB-PA63
1
CotB
275-595
PagD4
CotB-PagD4
cotC promoter
1
CotC
198-595
735
PagD4
CotC-PagD4

Figure 27

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Figure 28

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Figure 29

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Figure 30

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Figure 31

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Figure 32

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KSSKRSLKSSDDQSS

Figure 33

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Figure 34

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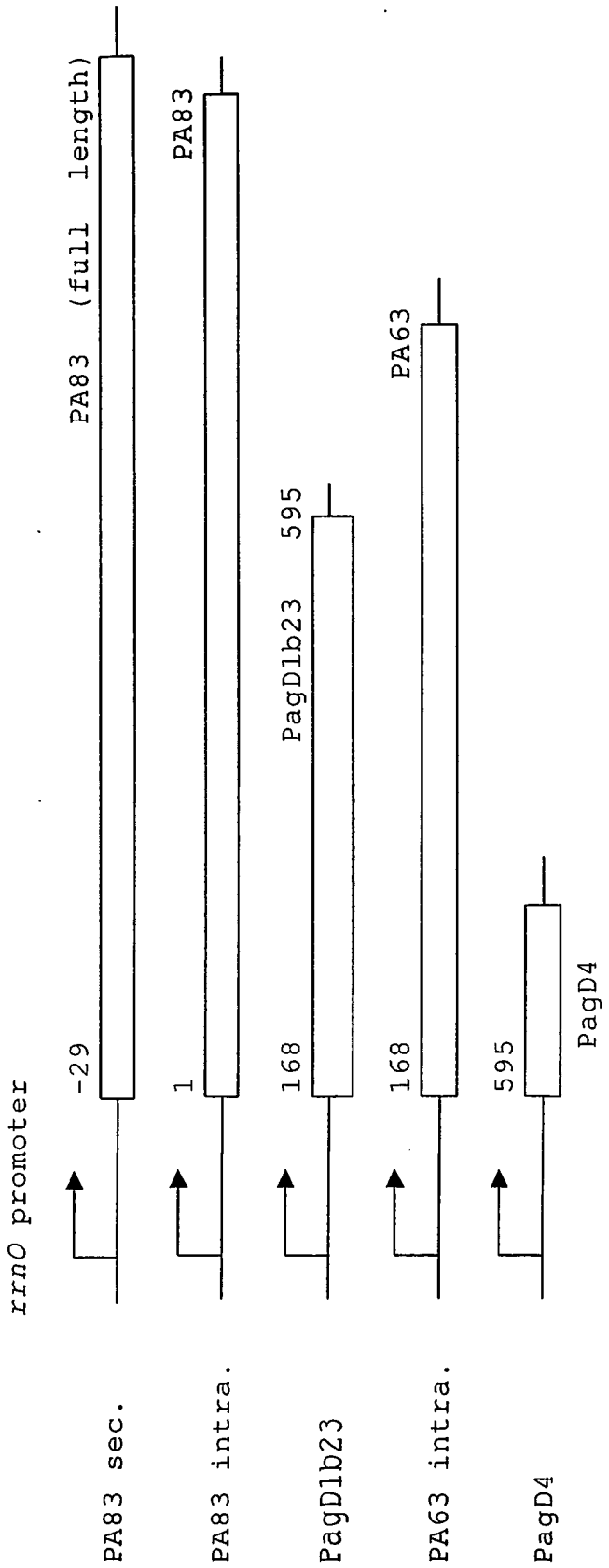
Figure 35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-29
PA83 (full length)
PA83 sec.
1
PA83
PA83 intra.
168
595
PagD1b23
PagD1b23
168
PA63
PA63 intra.
595
PagD4
PagD4

Figure 36

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Figure 37

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Figure 38

JPP430.ST25

SEQUENCE LISTING

<110> Royal Holloway University of London

<120> Improvements in or relating to Vaccination

<130> JPP237

<160> 17

<170> PatentIn version 3.3

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<211> 250
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<213> Bacillus anthracis

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Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
35 40 45

Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
50 55 60

Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
65 70 75 80

Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
85 90 95

Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
100 105 110

Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
115 120 125

Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
130 135 140

Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
145 150 155 160

Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
165 170 175

Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
180 185 190

Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser

195 200 205

Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
210 215 220

Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
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Gly Arg Ile Asp Lys Asn Val Ser Pro Glu
245 250

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Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr
20 25 30

Asp Ser Gln Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr
35 40 45

His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe
50 55 60

Asp Ile Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser
65 70 75 80

Thr Val Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp
85 90 95

Ala Glu Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala
100 105 110

Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu
115 120 125

Pro Thr Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile
130 135 140

Lys Ala Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr
145 150 155 160

Tyr Pro Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp
165 170 175

Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu
180 185 190

Glu Lys Thr Lys Gln Leu Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn
195 200 205

Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val Asp Thr Gly
210 215 220

Ser Asn Trp Ser Glu Val Leu Pro Gln Ile Gln Glu Thr
225 230 235

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<213> Bacillus anthracis

<400> 3

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Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr Thr Lys Pro
20 25 30

Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu
35 40 45

Pro Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp
50 55 60

Phe Asn Phe Asp Gln Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala
65 70 75 80

Glu Leu Asn Val Thr Asn Ile Tyr Thr Val Leu Asp Lys Ile Lys Leu
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Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys
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<213> Bacillus anthracis

<400> 4

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Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr Glu Gly
20 25 30

Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr
35 40 45

Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile Asn Asp
50 55 60

Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr
65 70 75 80

Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser
85 90 95

Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu Asn Thr
100 105 110

Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys
115 120 125

Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
130 135 140

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agtcgctttg agagaagcac acaaagttct ttgaaaacta aacaagacaa aacgtacctg 180

ttaattcatt tttataaatc gcacagcgat gtgcgtagtc agtcaaacta gggcctgcac 240

gacgcaggtc acacaggtgt cgccgcagga tgcggtgaac ttaacctgtt ctagaacaag 300

gaggtgagac ccatgggcag cagccatcat catcatcatc acagcagcgg cctggtgccg 360

cgcggcagcc atatggctag catgactggt ggacagcaaa tgggtcggga tccgaattcg 420

agctccgtcg acaagcttgc ggccgcactc gagcaccacc accaccacca ctgagatccg 480

gctgcc 486

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20 25 30

Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
35 40 45

Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
50 55 60

Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
65 70 75 80

Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
85 90 95

Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
100 105 110

Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
115 120 125

Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
130 135 140

Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
145 150 155 160

Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
165 170 175

Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
180 185 190

Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
195 200 205

Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
210 215 220

Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
225 230 235 240

Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
245 250 255

Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
260 265 270

Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Gln Thr Arg Thr
275 280 285

Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
290 295 300

Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
305 310 315 320

Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
325 330 335

Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
340 345 350

Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
355 360 365

Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
370 375 380

Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln
385 390 395 400

Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
405 410 415

Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
420 425 430

Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
435 440 445

Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
450 455 460

Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
465 470 475 480

Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
485 490 495

Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
500 505 510

Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
515 520 525

Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
530 535 540

Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
545 550 555 560

Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Val Thr Asn Ile Tyr Thr
565 570 575

Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
580 585 590

Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp
595 600 605

Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
610 615 620

Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
625 630 635 640

Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
645 650 655

Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
660 665 670

Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
675 680 685

Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
690 695 700

Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
705 710 715 720

Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
725 730 735

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<211> 568
<212> PRT
<213> Bacillus anthracis

<400> 7

Ser Thr Ser Ala Gly Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile
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Pro Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys
20 25 30

Arg Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly
35 40 45

Leu Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp
50 55 60

Pro Tyr Ser Asp Phe Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val
65 70 75 80

Ser Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His
85 90 95

Val Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr

100 105 110

Gln Asn Thr Asp Ser Gln Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr
115 120 125

Ser Arg Thr His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala
130 135 140

Ser Phe Phe Asp Ile Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser
145 150 155 160

Asn Ser Ser Thr Val Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu
165 170 175

Arg Thr Trp Ala Glu Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg
180 185 190

Leu Asn Ala Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr
195 200 205

Asn Val Leu Pro Thr Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu
210 215 220

Ala Thr Ile Lys Ala Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro
225 230 235 240

Asn Asn Tyr Tyr Pro Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala
245 250 255

Gln Asp Asp Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr Asn Gln Phe
260 265 270

Leu Glu Leu Glu Lys Thr Lys Gln Leu Arg Leu Asp Thr Asp Gln Val
275 280 285

Tyr Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val
290 295 300

Asp Thr Gly Ser Asn Trp Ser Glu Val Leu Pro Gln Ile Gln Glu Thr
305 310 315 320

Thr Ala Arg Ile Ile Phe Asn Gly Lys Asp Leu Asn Leu Val Glu Arg
325 330 335

Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr Thr Lys Pro
340 345 350

Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu
355 360 365

Pro Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp

370 375 380

Phe Asn Phe Asp Gln Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala
385 390 395 400

Glu Leu Asn Val Thr Asn Ile Tyr Thr Val Leu Asp Lys Ile Lys Leu
405 410 415

Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe His Tyr Asp
420 425 430

Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala
435 440 445

His Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Leu Asn Ile
450 455 460

Asp Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu
465 470 475 480

Asp Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg Tyr Asp Met Leu
485 490 495

Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile Asp Phe Lys
500 505 510

Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys
515 520 525

Val Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser
530 535 540

Glu Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe
545 550 555 560

Ser Lys Lys Gly Tyr Glu Ile Gly
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tctaatattc atgaaaagaa aggattaacc aaatataaat catctcctga aaaatggagc 180

acggcttctg atccgtacag tgatttcgaa aaggttacag gacggattga taagaatgta 240

tcaccagagg caagacaccc ccttgtggca gcttatccga ttgtacatgt agatatggag 300

aatattattc tctcaaaaaa tgaggatcaa tccacacaga atactgatag tcaaacgaga 360

acaataagta aaaatacttc tacaagtagg acacatacta gtgaagtaca tggaaatgca 420

gaagtgcatg cgtcgttctt tgatattggt gggagtgtat ctgcaggatt tagtaattcg 480

aattcaagta cggtcgcaat tgatcattca ctatctctag caggggaaag aacttgggct 540

gaaacaatgg gtttaaatac cgctgataca gcaagattaa atgccaatat tagatatgta 600

aatactggga cggctccaat ctacaacgtg ttaccaacga cttcgttagt gttaggaaaa 660

aatcaaacac tcgcgacaat taaagctaag gaaaaccaat taagtcaaat acttgcacct 720

aataattatt atccttctaa aaacttggcg ccaatcgcat taaatgcaca agacgatttc 780

agttctactc caattacaat gaattacaat caatttcttg agttagaaaa aacgaaacaa 840

ttaagattag atacggatca agtatatggg aatatagcaa catacaattt tgaaaatgga 900

agagtgaggg tggatacagg ctcgaactgg agtgaagtgt taccgcaaat tcaagaaaca 960

actgcacgta tcatttttaa tggaaaagat ttaaatctgg tagaaaggcg gatagcggcg 1020

gttaatccta gtgatccatt agaaacgact aaaccggata tgacattaaa agaagccctt 1080

aaaatagcat ttggatttaa cgaaccgaat ggaaacttac aatatcaagg gaaagacata 1140

accgaatttg attttaattt cgatcaacaa acatctcaaa atatcaagaa tcagttagcg 1200

gaattaaacg taactaacat atatactgta ttagataaaa tcaaattaaa tgcaaaaatg 1260

aatattttaa taagagataa acgttttcat tatgatagaa ataacatagc agttggggcg 1320

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attaatccta gtgagaatgg ggatactagt accaacggga tcaagaaaat tttaatcttt 1680

tctaaaaaag gctatgagat aggataa 1707

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<212> DNA
<213> Bacillus anthracis

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gctcatagag aagtaattaa ttcgtcaaca gagggattat tgttaaatat tgataaggat 120

ataagaaaaa tattatcagg ttatattgta gaaattgaag atactgaagg gcttaaagaa 180

gttataaatg acagatatga tatgttgaat atttctagtt tacggcaaga tggaaaaaca 240

tttatagatt ttaaaaaata taatgataaa ttaccgttat atataagtaa tcccaattat 300

aaggtaaatg tatatgctgt tactaaagaa aacactatta ttaatcctag tgagaatggg 360

gatactagta ccaacgggat caagaaaatt ttaatctttt ctaaaaaagg ctatgagata 420

ggataa 426

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<211> 1281
<212> DNA
<213> Bacillus anthracis

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gaggtagaag gatatacggt tgatgtcaaa aataaaagaa cttttctttc accatggatt 120

tctaatattc atgaaaagaa aggattaacc aaatataaat catctcctga aaaatggagc 180

acggcttctg atccgtacag tgatttcgaa aaggttacag gacggattga taagaatgta 240

tcaccagagg caagacaccc ccttgtggca gcttatccga ttgtacatgt agatatggag 300

aatattattc tctcaaaaaa tgaggatcaa tccacacaga atactgatag tcaaacgaga 360

acaataagta aaaatacttc tacaagtagg acacatacta gtgaagtaca tggaaatgca 420

gaagtgcatg cgtcgttctt tgatattggt gggagtgtat ctgcaggatt tagtaattcg 480

aattcaagta cggtcgcaat tgatcattca ctatctctag caggggaaag aacttgggct 540

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Ser Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His
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Val Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr
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Gln Asn Thr Asp Ser Gln Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr
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Glu Thr Glu Glu Met Leu Asp Ala Asp Asp Phe His Ser Leu Ile Gly
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His Leu Ile Asn Gln Ser Val Gln Phe Asn Gln Gly Gly Pro Glu Ser
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165 170 175

His Lys Trp Val Ser Ile Asn Arg Gly Gly Pro Glu Ala Ile Glu Gly
180 185 190

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195 200 205

Glu Val Leu Arg Ile Tyr Pro Phe His Ile Lys Ser Ile Ser Leu Gly
210 215 220

Pro Lys Gly Ser Tyr Lys Lys Glu Asp Gln Lys Asn Glu Gln Asn Gln
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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/GB2005/000170

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/32 A61K39/07

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, BIOSIS, MEDLINE, EMBASE, WPI Data, PAJ, LIFESCIENCES, SCISEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROSSIER FABIEN ET AL: "Anthrax spores make an essential contribution to vaccine efficacy" INFECTION AND IMMUNITY, vol. 70, no. 2, February 2002 (2002-02), pages 661-664, XP002324746 ISSN: 0019-9567 abstract page 661 page 662, column 1, paragraphs 1,3 ----- -/--	1,2,5-9, 12-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 2005	02/05/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Voigt-Ritzer, H

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COHEN S ET AL: "ATTENUATED NONTOXINOGENIC AND NONENCAPSULATED RECOMBINANT BACILLUS ANTHRACIS SPORE VACCINES PROTECT AGAINST ANTHRAX" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 68, no. 8, August 2000 (2000-08), pages 4549-4558, XP002942521 ISSN: 0019-9567 page 4549 abstract table 1 page 4551, column 2, paragraph 4 - page 4552, column 1, paragraph 3	1,2,5-9, 12,14-19
X	BARNARD J P ET AL: "VACCINATION AGAINST ANTHRAX WITH ATTENUATED RECOMBINANT STRAINS OF BACILLUS ANTHRACIS THAT PRODUCE PROTECTIVE ANTIGEN" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 67, no. 2, February 1999 (1999-02), pages 562-567, XP002942520 ISSN: 0019-9567 page 562, column 2, paragraph 2 page 563, column 2, paragraphs 3,5 figure 4	1,2,5-9, 12,14-19
X Y	WO 02/00232 A (MAXYGEN, INC; GOLDMAN, STANLEY; LATHROP, STEPHANI, J; LONGCHAMP, PASCA) 3 January 2002 (2002-01-03) page 61, line 26 - line 32 examples 1,4,9 figure 6	1-3, 5-10, 12-24 4,11
Y	DUC L H ET AL: "BACTERIAL SPORES AS VACCINE VEHICLES" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 71, no. 5, May 2003 (2003-05), pages 2810-2818, XP009011619 ISSN: 0019-9567 abstract page 2810, column 2, paragraphs 1,3 page 2815, column 1, paragraph 1 page 2817, column 2, paragraph 2	1-3, 5-10, 12-24

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INTERNATIONAL SEARCH REPORT

Inter ıl Application No
PCT/GB2005/000170

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DUC L H ET AL: "Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 21, no. 27-30, 1 October 2003 (2003-10-01), pages 4215-4224, XP004462821 ISSN: 0264-410X page 4215	1-24
Y	ZEGERS N D ET AL: "Expression of the protective antigen of Bacillus anthracis by Lactobacillus casei: Towards the development of an oral vaccine against anthrax" JOURNAL OF APPLIED MICROBIOLOGY, vol. 87, no. 2, August 1999 (1999-08), pages 309-314, XP002324747 & 3RD INTERNATIONAL CONFERENCE ON ANTHRAX; PLYMOUTH, ENGLAND, UK; SEPTEMBER 7-10, 1998 ISSN: 1364-5072 the whole document	1-24
Y	WO 02/04646 A (THE SECRETARY OF STATE FOR DEFENCE; WILLIAMSON, ETHEL, DIANE; MILLER,) 17 January 2002 (2002-01-17) page 3, line 1 - line 3	1-24

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

Ir.....ational application No.
PCT/GB2005/000170

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 21-24
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 21-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte al Application No
PCT/GB2005/000170

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0200232 A	03-01-2002	AU 7300901 A	08-01-2002
		EP 1299115 A2	09-04-2003
		WO 0200232 A2	03-01-2002
		US 2002150594 A1	17-10-2002
		US 2003165538 A1	04-09-2003
WO 0204646 A	17-01-2002	AU 6930501 A	21-01-2002
		CA 2413045 A1	17-01-2002
		CN 1440459 A	03-09-2003
		EP 1301606 A1	16-04-2003
		WO 0204646 A1	17-01-2002
		JP 2004502460 T	29-01-2004
		US 2003170263 A1	11-09-2003
		ZA 200210206 A	17-03-2004